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SAĞLIK BİLİMLERİ ENSTİTÜSÜ
Tıbbi Biyoloji Anabilim Dalı

ENDOMETRİUM KARSİNOMUNDA ÖLÜMCÜL TRAIL LİGAND VE RESEPTÖR EKSPRESYON PROFİLİNİN TÜMÖR EVRELERİ İLE İLİŞKİLERİNİN BELİRLENMESİ

Çiğdem AYDIN ACAR

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Tez Danışmanı

Prof. Dr. Salih ŞANLIOĞLU

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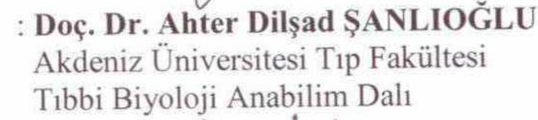

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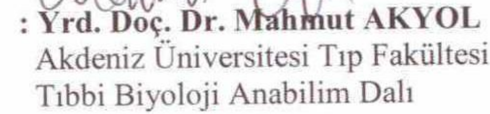

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2. Bisgin A, Terzioglu E, **Aydin C**, Yoldas B, Yazisiz V, Balci N, Bagci H, Gorczynski RM, Akdis CA. TRAIL Death Receptor-4, Decoy Receptor-1 and Decoy Receptor-2 expression on CD8+ T cells correlate with the disease severity in patients with rheumatoid arthritis. Sanlioglu S. *BMC Musculoskeletal Disorders.* 2010, 11:192

3. **Aydin C**, Sanlioglu AD, Karacay B, Ozbilim G, Dertsiz L, Ozbudak O, Akdis CA and Sanlioglu S. A DcR2 siRNA strategy employing three different siRNA constructs in combination defeats adenovirus transferred TRAIL resistance in lung cancer cells. *Human Gene Therapy.* 2007 Jan; 18: 39-50.

4. Sanlioglu AD, Dirice E, **Aydin C**, Erin E, Koksoy S and Sanlioglu S. Surface TRAIL decoy receptor-4 expression is correlated with TRAIL resistance in MCF7 breast cancer cells. *BMC Cancer.* 2005 25;5(1):54

ÖZET

Endometrium kanseri dünyada en sık görülen jinekolojik kanser türüdür ve kadınlarda meme, akciğer ve kolon kanserinden sonra dördüncü sırada yer almaktadır. TRAIL ve TRAIL reseptör ekspresyon profili son yıllarda kanserde prognostik belirteç olarak kullanılma potansiyeli üzerinde en çok araştırma yapılan konulardan biridir. TRAIL ölüm ligandı normal hücrelere zarar vermeksizin kanser hücrelerinde seçici olarak apoptozisi indükleyebilmektedir. TRAIL'ın membrana bağlı dört reseptörü tanımlanmıştır ki, bunlardan ikisi apoptozise aracılık eden ölüm reseptörleri, TRAIL-R1 (DR4) ve TRAIL-R2 (DR5) ve diğer ikisi ise apoptozisi inhibe eden dekoy reseptörlerdir, TRAIL-R3 (DcR1) ve TRAIL-R4 (DcR2). Fakat, TRAIL ve TRAIL reseptörlerinin endometrial karsinogeneze nasıl katkı sağladığı bilinmemektedir. Bu nedenle, bu çalışmada, prognostik belirteç olarak kullanılabilme potansiyelini değerlendirmek ve TRAIL aracılı gen tedavi yaklaşımının uygulanabilirliğini önceden tahmin edebilmek için endometrial karsinogenezde TRAIL ligand ve reseptör ekspresyon profili araştırılmıştır. Bu bağlamda, immunohistokimya yöntemi kullanılarak normal endometrium (n=18), endometrial hiperplazi (n=27) ve endometrial karsinoma dokularında (n=100) TRAIL ve reseptörlerinin ekspresyon profili analiz edilmiştir. Ayrıca, tüm dokularda apoptotik indeksi değerlendirmek üzere TUNEL yöntemi kullanılmıştır. Değerlendirilen endometrial karsinoma örneklerinde, normal endometrial dokular ile karşılaştırıldığında TRAIL ligand ve DR4 ölüm reseptör ekspresyonlarında azalma, DR5 ölüm reseptörü ve her iki dekoy reseptör (DcR1 ve DcR2) ekspresyonlarında ise artış gözlenmiştir. Ayrıca, değerlendirilen endometrial hiperplazi örneklerinin endometrial karsinomada gözlenen TRAIL ve TRAIL reseptör ekspresyon profiline sahip olduğu gözlenmiştir. Diğer taraftan, yapılan değerlendirmelerde TRAIL/TRAIL reseptör ekpresyon profili ile bilinen prognostik faktörler (tümör evresi, histolojik derece ve myometrial invazyon derinliği) arasında bir korelasyon belirlenememiştir. Sonuç olarak, endometrial karsinomalı hastalarda belirlenen TRAIL ve reseptörlerinin özgün ekspresyon profili TRAIL sinyalleşmesinin endometrial karsinoma gelişiminde önemli olabileceğini işaret etmektedir.

Anahtar Kelimeler: Endometrial Karsinoma, TRAIL, TRAIL reseptörleri

ABSTRACT

Endometrial cancer is the most common gynecologic malignancy in the world and the fourth most common maligancy in women after breast, colorectal and lung cancer. TRAIL and TRAIL receptor profile has recently been tested for its potential to be used as a prognostic marker in cancer. TRAIL death ligand selectively induces apoptosis in cancer cells without damaging healthy cells. Four membrane bound receptors for TRAIL have been identified, two apoptosis-mediating receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), and two apoptosis inhibiting receptors, TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2). However, how TRAIL and TRAIL receptors contribute to endometrial carcinogenesis is not known. Thus, we investigated TRAIL and TRAIL receptor expression profiles during endometrial carcinogenesis to evaluate its potential as a prognostic marker and to predict the feasibility of TRAIL-mediated gene therapy approach. To test this, the expression profile of TRAIL and its receptors were analyzed and compared in normal endometrium (n=18), endometrial hyperplasia (n=27) and endometrial carcinoma samples (n=100) using immunohistochemistry. In addition, TUNEL assay was performed to determine the apoptotic index on all tissues. A decrease in TRAIL ligand and DR4 death receptor expression but an increase in DR5 death receptor and both of the decoy receptor (DcR1 and DcR2) expressions were observed in endometrial carcinoma samples compared with normal endometrial tissues. In addition, endometrial hyperplasia samples displayed TRAIL and TRAIL receptor expression profile similar to that of endometrial carcinoma samples. On the other hand, TRAIL/TRAIL receptor expression profiles did not correlate with any known prognostic factors (tumor stage, tumor grade and depth of myometrial invasion). As a result, distinctive expression profiles of TRAIL and its receptors in patients with endometrial carcinoma suggests that TRAIL signaling might be important during the endometrial carcinoma development.

Key Words: Endometrial Carcinoma, TRAIL, TRAIL receptors.

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SİMGELER ve KISALTMALAR DİZİNİ

TNF	**:** Tumor Necrosis Factor (Tümör Nekrozis Faktör)
TRAIL	**:** Tumor Necrosis Factor-related Apoptosis-Inducing Ligand (Tümör Nekrozis Faktör ilişkili Apoptozis İndükleyici Ligand)
DcR	**:** Decoy Receptor (Yalancı Reseptör)
DR	**:** Death Receptor (Ölüm Reseptörü)
FADD	**:** Fas associated death domain (Fas ilişkili ölüm domaini)
DISC	**:** Death-inducing signaling complex (Ölüm indükleyici sinyal kompleksi)
DD	**:** Death domain (Ölüm Domaini)
DED	**:** Death effector domain (Ölüm Effektör Domaini)
IAP	**:** Inhibitor of Apoptosis Protein (Apoptozis İnhibe Edici Protein)
WHO	**:** World Health Organization (Dünya Sağlık Örgütü)
ISGP	**:** International Society of Gynecological Pathologists (Uluslararası Jinekolojik Patologlar Derneği)
FIGO	**:** International Federation of Gynecology and Obstetrics (Uluslararası Jinekoloji ve Obstetrik Derneği)
EIC	**:** Endometrial Intraepithelial Carcinoma (Endometrial İntraepitelyal Karsinoma)
TUNEL	**:** Terminal deoxynucleotidyl transferase (Tdt)-mediated dUTP-biotin nick end-labeling
TdT	**:** Terminal deoksinükleotidil transferaz
Aİ	**:** Apoptotik İndeks
LOH	**:** Loss of Heterozygosity (Heterozigotluğun kaybı)
MSI	**:** Mikrosatellit İnstabilite
DNA	**:** Deoksiribonükleik asit
SEM	**:** Standard error of mean
DAB	**:** 3,3'-Diaminobenzidine
PSA	**:** Prostat Spesifik Antijen
FAK	**:** Fokal Adezyon Kinaz
BPH	**:** Benign Prostatik Hiperplazi
OCPCa	**:** Organ confined prostate carcinoma (Organa sınırlı prostat karsinoma)
APCa	**:** Advanced prostate cancer
PI3K	**:** Phosphatidylinositol 3-kinases
PIP3	**:** Phosphatidylinositol (3,4,5)-trisphosphate
PTEN	**:** Phosphatase and tensin homolog
PBMC	**:** Peripheral blood mononuclear cell
NK	**:** Natural killer hücreler
IFN	**:** Interferon
CFLIP	**:** FLICE-inhibitory protein
HNPCC	**:** Herediter nonpolipozis kolorektal kanser

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GİRİŞ VE AMAÇ

Endometrium karsinomu son yıllarda dünyada özellikle gelişmiş ülkelerde belirgin bir artış eğilimi göstermekte olup gelişmiş ülkelerde en sık görülen kadın genital sistem kanseri haline gelmiştir. Kadınlarda meme, akciğer ve kolorektal karsinomlardan sonra en sık görülen malign tümör tipidir. Yüksek görülme sıklığına karşılık ölüme neden olan kanserler arasında alt sıralarda yer almaktadır ve kanser ölümlerinin 7. en sık nedenidir. Endometrium kanseri genelde postmenopozal kadınlarda görülen ve yaş arttıkça seyri kötüleşen bir hastalıktır. Ancak, hastaların %25'i premenopozal, hatta % 5'i 40 yaş öncesinde, % 70'i postmenopozal dönemde görülür. Genellikle 50-65 yaşları arasında görülmesine rağmen ortalama görülme yaşı 60'dır. Endometrioid adenokarsinomlar ise en sık görülen endometrium kanser tipidir. Vakaların neredeyse tamamı hormona bağımlıdır; obezite, ekzojen hormon alımı ve yüksek östrojen seviyesi ile ilişkilidir. Bu tip endometriyum karsinomları, endometriyal hiperplaziden gelişmektedir. Karsinogenezinde onkojen mutasyonları ve tümör baskılayıcı genlerin kaybıyla oluşan bir dizi genetik değişikliğin önemli rolü vardır. Proliferasyon ve apoptozis arasındaki denge bozuldukça, endometriyum hiperplaziden karsinoma kadar değişiklikler gösterebilir. Endometriyumun nadir görülen seröz karsinomları ise daha çok endometriyal intraepiteliyal neoplaziden gelişmektedir. Endometrium kanserli vakaların dörtte üçü erken tanı almaktadır ve bu evrede cerrahi müdahale sonucu elde edilen 5 yıllık sağkalım oranı % 80'dir. Ancak, ileri evre endometrial kanserli hastaların önemli bir kısmı mevcut tedavi yöntemlerine rağmen tedavi edilememektedir.

TRAIL ölüm ligandı, ilk kez 1995 yılında TNF süper ailesinin yeni bir üyesi olarak tanımlanmıştır. TRAIL ile etkileşim kurabilen beş reseptör mevcuttur; TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4 ve osteoprotegrin. TRAIL-R1 ve TRAIL-R2 apoptozisi indükleyen gerçek ölüm reseptörleri olarak fonksiyon görürken, TRAIL-R3 ve TRAIL-R4 apoptozisi indükleyememekte ancak dekoy reseptör olarak görev yapmaktadır. TRAIL'ın sadece kanser hücrelerinde apoptozisi indüklemesi, normal hücrelerde aynı etkiyi göstermemesi kanser tedavisinde kullanımını gündeme getirmiştir. TRAIL'ın kanserde terapötik olarak kullanımına yönelik yapılan çalışmalarda ise tümör hücrelerinin önemli bir kısmında TRAIL'a karşı dirençlilik tespit edilmiştir. Fakat ortaya çıkan bu dirençliliğin mekanizması henüz tam olarak bilinmemektedir. Bununla birlikte yapılan çalışmalarda TRAIL ligand ve reseptör profillerinin kanser tipleri arasında farklılık gösterdiği saptanmıştır.

Yapılan çalışmalar, TRAIL ligand ve reseptör ekspresyon profillerinin kanser prognozunu belirlemede belirteç olarak kullanılabileceğini işaret etmektedir. Bununla birlikte, literatüre bakıldığında endometrium kanserli hastalara ait tümör dokularında TRAIL ligand ve TRAIL reseptör profilinin ve apoptozis üzerindeki etkilerinin aydınlatılmasına yönelik bir çalışma bulunmamaktadır. Özellikle TRAIL

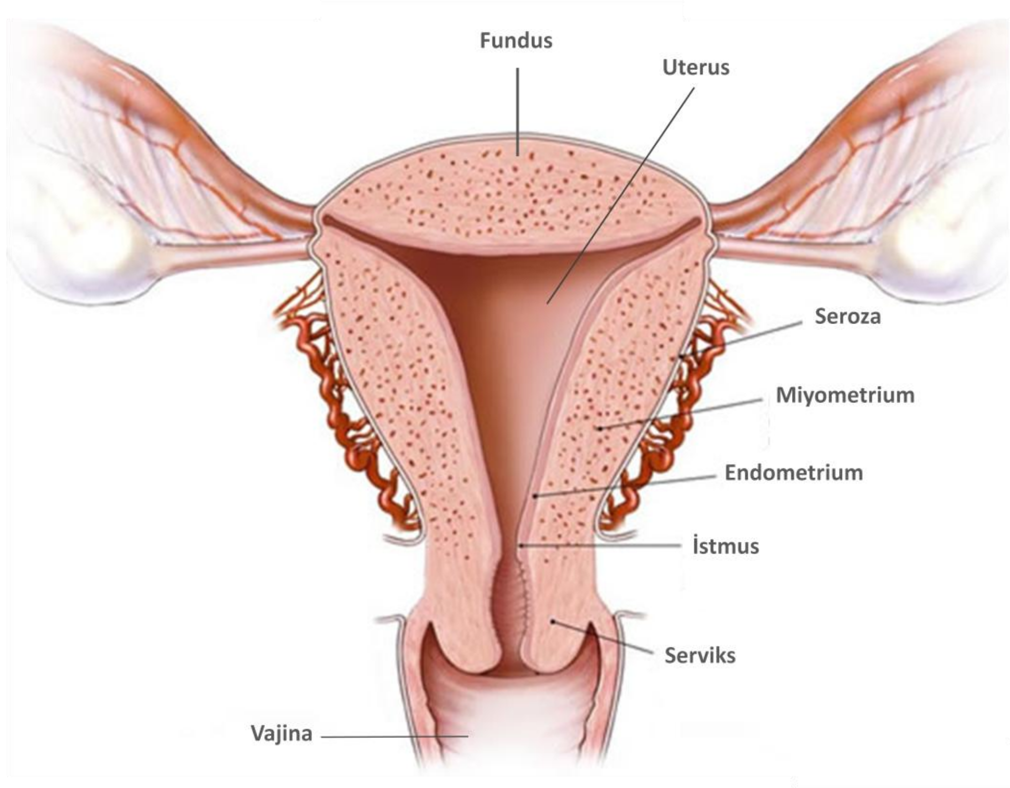
ligand ve reseptör profilinin tümör evreleri ile ilişkisinin belirlenmesinin hastalığın seyrini belirlemede bir belirteç olabileceği düşünülmektedir. Bu sebeple çalışmamızda, endometrioid karsinomlu ve endometrial hiperplazili hasta dokularında TRAIL ligand ve reseptör ekspresyon profilinin ortaya çıkarılarak kanser gelişimi ve prognozu üzerinde etkisinin olup olmadığının belirlenmesi amaçlanmaktadır. Ayıca, endometrial kanserli hastalarda TRAIL ligand ve reseptör ekspresyon profilinin belirlenmesinin gelecekte TRAIL odaklı hastaya spesifik tedavi protokollerinin oluşumuna katkı sağlanması beklenmektedir.

GENEL BİLGİLER

2.1. Uterus Anatomisi

Dişi üreme sistemini meydana getiren organlar dış ve iç üreme organları olarak iki kısımda incelenmektedir. Dış üreme organlarının tümüne birden vulva adı verilir. İç üreme organları ise vajina, uterus, ovaryumlar ve fallop tüplerinden oluşmaktadır [1-3].

Uterus, alt bölümünü serviksin oluşturduğu, armut biçiminde, mesane ve rektum arasına yerleşmiş musküler bir organdır. Uterus boşluğu üçgen şeklindedir. Üstte fallop tüpleri ile periton boşluğu, altta ise vajinal kanal yoluyla dış ortamla bağlantılıdır. Uterus anatomik olarak fundus, korpus, istmus ve serviks olmak üzere dört bölümde incelenir. Uterusun en üst kısmı olan fundus, tuba uterinaların uterusa açıldıkları seviyenin üzerinde kalan bölümdür. Korpus, tuba uterinaların uterusa açıldığı seviyeden istmusa kadar uzanan bölgedir ve uterusun esas parçası kabul edilmektedir. Vajina ön duvarında sonlanan en alt parçaya serviks, korpus ve serviks arasında kalan 0,5 cm uzunluğundaki bölgeye ise istmus adı verilmektedir (Şekil 2.1) [1-3].



Şekil 2.1. Uterus anatomisi [4].

Uterus dış yüzeyi, seroza tabakasını oluşturan periton ile kaplıdır. Seroza tabakasının altında düz kaslardan oluşan müsküler tabaka (miyometrium) yer almaktadır. Miyometriumun altında ise stroma adı verilen, gevşek bağ dokusu ve hücrelerle çevrili tübüler salgı bezlerinden oluşan endometrium tabakası yer alır. Endometrium, stratum basale ve endometrial glandların bulunduğu stratum fonksiyonaleden oluşur. Fonksiyonel tabaka, over hormonlarının (östrojen ve progesteron) etkisiyle değişime uğrar. Bu değişim, puberteden menopoza kadar sürer. Normalde yaklaşık 28 günde bir görülen menstrüasyon, endometriyal değişikliğin en belirgin özelliğidir [1-3].

2.2 Endometrial Hiperplaziler

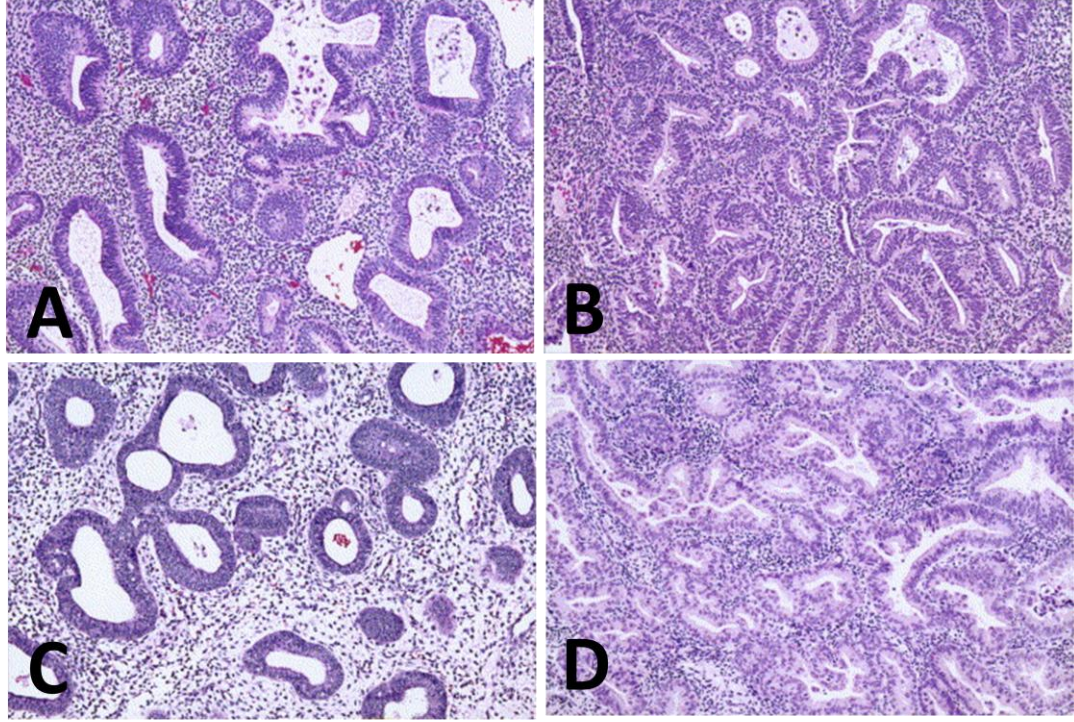
İlk kez 1900 yılında Dr. Cullen endometriumda endometial kanserin öncüsü olduğunu düşündüğü histolojik bir yapı tanımladı. Cullen'in açtığı yoldan ilerleyen diğer araştırmacılar (1932'de Taylor ve 1936'da Novak ve Yui) bu tabloya endometrial hiperplazi adını verdiler ve 1947 yılında Dr. Gusberg bu hastalığın sınıflamasını yaptı [5].

Endometrial hiperplazi, normal proliferatif endometriuma kıyasla artmış bez ve stroma oranı, düzensiz şekil ve boyutlardaki bez proliferasyonu olarak tanımlanır [6]. Bu olay yaygındır fakat bütün endometriumu tutması gerekmez. Bu lezyonların bir kısmı spontan olarak veya tedavi ile gerileyebilmekte, bir kısım hastada hiperplazi persiste kalmakta, az bir kısım hastada ise endometrial adenokarsinomaya dönebilmektedir. Endometrial hiperplazi tanısı alan hastalarda en sık karşılaşılan neden karşılanmamış östrojene maruz kalınmasıdır. Karşılanmamış östrojene maruz kalmanın en sık nedeni ise anovulasyondur. Östrojenler, endometrial epitel ve stroma hücrelerinin mitotik aktivitelerini indüklemektedir. Östrojenik etki altındaki endometrium prolifere olur ve glandular epitelyum gerçek olmayan sekilde katmanlaşır. Sonuç olarak, progesteron tarafından karşılanmamış sürekli östrojen stimulasyonu endometrial hiperplaziye yol açar [5].

Endometrial hiperplaziler için WHO'nun yaptığı sınıflandırma iki kritere dayanmaktadır; glandüler yoğunluk ve nüklear görünüm [7]. Bu kriterlere dayalı olarak WHO'nun endometrial hiperplaziler için yapmış olduğu sınıflandırma Tablo 2.1'de verilmiştir.

Tablo 2.1. Endometrial hiperplazilerin WHO sınıflandırması [8].

Atipik olmayan hiperplaziler (Tipik)
Basit atipisiz hiperplazi
Kompleks atipisiz hiperlazi
Atipik hiperplaziler
Basit atipik hiperplazi
Kompleks atipik hiperplazi



Şekil 2.2. Endometrial Hiperplazi tipleri A) Basit atipisiz hiperplazi B) Kompleks atipisiz hiperplazi C) Basit atipik hiperplazi D) Kompleks atipik hiperplazi [8].

Basit hiperplazi; genişlemiş yuvarlak ya da hafif düzensiz şekillerdeki kistik bezler ve glandüler kalabalıklaşma olmaksızın artmış bez stroma oranı ile karakterizedir. Bezler genelde proliferatiftir ve polipoid gelişim gözlenebilir. Kompleks hiperplazide ise sitolojik atipi olmadığı halde yaygın glandüler yapısal atipi veya kalabalıklaşma gözlenir. Eldiven parmağı, testere dişli, papiller görünüm, stromada bezlerde yoğun kalabalıklaşma, sırt sırta verme görünümü dikkati çeker. Atipik hiperplaziler basit veya kompleks yapısal özelliklere sahiptir. Bezlerin kompleks yapısına ve kalabalık görünümüne ek olarak tanı koydurucu sitolojik atipi mevcuttur (Şekil 2.2) [9-11].

Endometrial hiperplaziler, sitolojik atipinin varlığına ve ağırlığına bağlı olarak adenokarsinomaya dönüşme riski taşımaktadır [12]. Endometrioid kanser vakalarının pek çoğu, atipik endometrial hiperplaziden gelişen endometrioid karsinomalardır. Kanser gelişim olasılığı hiperplazi derecesi ile ilişkilidir. Kanser progresyon riski basit atipisiz hiperplazide 1-kat, kompleks atipisiz hiperplazide 3-kat, basit atipik hiperplazide 8-kat ve kompleks atipik hiperplazide ise 29-kat artmaktadır [13-14].

2.3 Endometrium Kanseri

Endometrium kanseri, uterusun iç tabakasını oluşturan endometriumdaki hücrelerin kontrolsuz çoğalması sonucu oluşmaktadır. Bu yüzyılın ikinci yarısında pap smear taramalarının başarısına bağlı olarak serviks kanseri insidansında belirgin azalma söz konusu olmuş, bu da bilhassa gelişmiş ülkelerde endometrium kanserinin en sık görülen jinekolojik kanser haline gelmesine yol açmıştır. Genel olarak bakıldığında ise, endometrium kanserinin, akciğer, meme ve kolon kanserinden sonra kadınlarda gözlenen en sık dördüncü kanser olduğu fark edilmektedir. [15]. Hastalık insidansının kıtalara göre değişkenlik gösterebildiği, özellikle de gelişmiş ülkelerde daha yüksek olduğu bilinmektedir. Nitekim endometrial kanser Kuzey Amerika ve Avrupa'lı kadınlarda gözlenen tüm kanser vakalarının yaklaşık %8-10'unu, Afrika ve Asya'lı kadınlarda ise %2-4'ünü oluşturmaktadır [16].

Yapılan kanser istatistiklerine göre 2010'da ABD'de 43.470 yeni olguya tanı konulmuş ve 7.950 kadın endometrium karsinomundan dolayı hayatını kaybetmiştir. Yüksek insidansına karşılık endometrial karsinom, kadınlardaki kanser ölümlerinin % 3'ünden sorumludur ve ölüme neden olan kanserler arasında sekizinci sırada yer almaktadır. [17].

Endometrium karsinomu olgularının % 95'i 40 yaş üzerindedir. % 70-80'i postmenopozal, % 25'i premenopozal dönemde görülür ve vakaların sadece % 5'i 40 yaşından daha gençtir. Genellikle 50-65 yaşları arasında görülür ve ortalama görülme yaşı 61'dir [18-19].

2.3.1 Endometrium Kanserlerinin Tanısı ve Risk Faktörleri

Endometrial karsinomların %90'dan fazlasında düzensiz veya post-menapozomal vajinal kanama mevcuttur. Fakat post-menopozomal kanamalı hastaların sadece %20'sinde kanama malignant orjinlidir ve bunların %50'si endometrial karsinoma nedeniyledir [19]. Endometrium kanser vakalarının %5' i ise asemptomatiktir. Genel populasyonda endometrium kanserinin belirlenmesi için bir tarama programı mevcut değildir. Tüm vakalarda değerlendirme ilk olarak transvajinal ultrason ile yapılmaktadır. Endometrium kalınlığı 4 mm'den fazla veya normal endometrial kalınlığa sahip olduğu halde kanama şikayeti bulunan tüm postmenapozal hastalardan endometrial biyopsi veya küretaj ile örnek alınarak değerlendirilmekte, histereskopi ile de inceleme yapılabilmektedir. Endometrial kanser tanısında bilgisayarlı tomografi, manyetik rezonans görüntüleme ve pozisyon emisyon tomografisi gibi görüntüleme yöntemleri de gerektiğinde kullanılabilmektedir [19-21].

Endometrium kanseri için tanımlanmış risk faktörleri endometrioid (Tip 1) formuna ait olmakla birlikte, büyük oranda karşılanmamış östrojene maruz kalma ile ilişkilidir. Yüksek düzeyde uzun süreli karşılanmamış östrojen endometriumda mitotik aktiviteyi uyarır ve endometrial hiperplaziye yol açarak daha uzun sürede maligniteye dönüşüm gösterebilir. Tablo 2.2'de endometrial kanser için tanımlanmış risk faktörleri verilmiştir.

Tablo 2.2. Endometrium kanseri risk faktörleri [22].

Faktör	*Risk Oranı (%)*
Uzun süreli, yüksek doz östrojen kullanımı, Karşılanmamış menopozal östrojen	10-20
Yüksek dozlarda Tamoksifen kullanımı	3-7
Östrojen üreten tümör	> 5
Obezite	
BMI 30-34 kg/m^2	1.7
BMI 35-39 kg/m^2	4.3
BMI ≥ 40 kg/m^2	6.4
Nulliparite	3
Diyabet, Hipertansiyon, Tiroit, Safra Kesesi Hastalığı	1.3-3
İleri yaş	2-3
İnfertilite	2-3
Geç Menopoz	2-3
Erken Menarş	1.5-2
Menstrual düzensizlik	1.5
Beyaz ırk	2
Oral kontraseptifler	0.3-0.5
Sigara kullanımı	0.5

2.3.2 Endometrium Kanserlerinin Patolojisi ve Evrelendirilmesi

Bokhman 1983 yılında endometrial karsinomaları 2 tip olarak sınıflandırmıştır [23]. Tip 1 olarak isimlendirilen birinci grup, temel olarak endometrioid karsinomalardan oluşmaktadır. Tip 1 endometrial karsinom östrojen ilişkilidir ve genel olarak endometrial hiperplaziden kaynaklanmaktadır. Sıklıkla düşük grade malignansi gösterirler [24-25]. Bu tümörler pre- ve post menopozal kadınlarda endometrial karsinomların yaklaşık %80'ini oluşturur ve vakaların %74'ünde 5 yıllık sağkalım oranı ile iyi prognoz söz konusudur [15]. Endometrioid karsinomun sekretuar karsinoma ve villoglandular karsinom da dahil olmak üzere birkaç alt tipi veya varyantları tanımlanmıştır [26].

Tip 2 endometrial kanserler, seröz veya berrak hücreli endometrial karsinomalardan (non-endometrioid karsinomalar) oluşmaktadır. Bu kanserler östrojen ile ilişkili değildir ve endometrial hiperplaziden köken almazlar. Nadiren endometrial intraepitelyal karsinomlardan (EIC) kaynaklanan endometrial polipler veya prekanseröz lezyonlar gözlenir. Genellikle yüksek grade, derin myometrial invazyon ve lenfatik yayılım gösterirler. 5 yıllık sağ kalım %27-42 arasındadır ve kötü prognoza sahiptirler. Tip 2 Endometrial karsinoma esas olarak post-menopozal kadınlarda ortaya çıkar ve günümüzde risk faktörleri sadece yaş ve pelvik ışın

uygulaması olarak düşünülmektedir [15, 27]. Tablo 2.3'de her iki tipe ait klasik özellikler listelenmiştir.

Tablo 2.3. Endometrial karsinoma tipleri [10].

	Tip I	Tip II
Menopozal durum	Premenopozal ve perimenopozal	Postmenopozal
Östrojen ilişkisi	Var	Yok
Östrojen veya Progesteron Reseptörleri	Var	Yok
Bitişik endometriumun Histolojisi	Hiperplastik	Atrofik/sistik polip
Öncü lezyon	Atipik Hiperplazi	EIC
Obezite	Var	Yok
Doğum	Nulipar	Multipar
Grade	Düşük	Yüksek
Histolojik alt tip	Endometrioid	Seröz karsinoma Berrak-hücreli karsinoma
Klinik	Ağrısız	Agresif

Endometrioid karsinomların % 30'u atrofik endometrium ile ilişkili olduğundan dolayı iki kategori ile de çakışmaktadır. Ayrıca, bileşik endometrioid/seröz karsinomalar mevcuttur ve vakaların % 46'sı endometrial hiperplazi ile ilişkilendirilmektedir.

Endometrial karsinomlarda Dünya Sağlık Örgütü'nün (WHO) ve International Society of Gynecological Pathologists'in (ISGP) önerdiği sınıflama kullanılmaktadır (Tablo 2.4)

Tablo 2.4. Endometrial karsinomlarda sınıflandırma (WHO ve ISGP)[28]

Endometrioid adenokarsinom
Villoglandüler
Sekretuar
Silyalı hücreler
Skuamöz diferansiyasyonlu
Müsinöz karsinoma
Seröz karsinoma
Berrak hücreli karsinoma
Bileşik (Mikst) karsinoma
Skuamöz- hücreli karsinoma
Transisyonel-hücreli karsinoma
Küçük –hücreli karsinoma
Andiferansiye karsinom

Evrelendirme Endometrioid karsinomlarda prognostik olarak önemlidir. Tablo 2.5 ve 2.6'de sırasıyla 1988 ve 2009 International Federation of Gynecology and Obstetrics (FIGO) evrelendirme sistemine göre endometrial karsinomların evrelendirilmesi yapılmıştır [29-32].

Tablo 2.5. Endometriyum kanseri cerrahi sınıflama (FIGO 1988)

Evre	*Tanımlama*
Evre I	**Tümör endometriyuma sınırlı**
IA	**Tümör endometriyuma sınırlı**
IB	**Myometriyal invazyon <1/2**
IC	**Myometriyal invazyon >1/2**
Evre II	**Tümör serviks ve endometriumu invaze etmiş**
IIA	**Tümör endoservikal glandüler yapıyı invaze etmis**
IIB	**Tümör endoservikal stromal yapıyı invaze etmis**
Evre III	**Tümörün lokal ve/veya bölgesel yayılımı**
IIIA	**Tümör seroza ve/veya adnekse yayılmış ve/veya periton sitolojisi pozitif**
IIIB	**Tümör Vajinaya yayılmış**
IIIC	**Pelvik ve/veya paraaortik lenf nodu metastazı**
Evre IV	**İntra-abdominal veya ekstra-abdominal metastazlar**
IVA	**Tümörün mesane veya bağırsak mukozasına invazyonu**
IVB	**Uzak metastazlar (intraabdominal metastazlar ve/veya inguinal lenf nodları)**

Endometrium Karsinomunda FIGO'nun Grade (Derece) Tanımlaması (1988)

G1(İyi diferansiye): Nonskuamoz veya nonmoruler solid büyüme şekilleri ≤ % 5
G2(Orta diferansiye): Nonskuamoz veya nonmoruler solid büyüme şekilleri %6–50
G3(Kötü diferansiye): Nonskuamoz veya nonmoruler solid büyüme şekilleri > % 50
Yapısal grade ile uyumsuz belirgin nükleer atipi grade'i 1 puan yükseltir.

Tablo 2.6. Endometriyum kanseri cerrahi sınıflama (FIGO 2009)

Evre	*Tanımlama*
Evre I	**Tümör endometrium sınırlı**
IA	**Myometriyal invazyon yok veya <1/2**
IB	**Myometriyal invazyon ≥1/2**
Evre II	**Tümör servikal stromayı kapsar**
Evre III	**Tümörün lokal ve/veya bölgesel yayılımı**
IIIA	**Tümör seroza ve/veya adnekse yayılmış**
IIIB	**Vajinal ve/veya parametrial yayılım**
IIIC	**Pelvik ve/veya paraaortik lenf nodu metastazı**
IIIC1	**Pozitif pelvik nodlar**
IIIC2	**Pozitif lenf nodlu ve nodsuz pozitif para-aortik lenf nodları**
Evre IV	**Tümörün mesane ve/veya bağırsak mukozasına ve/veya uzak metastazları**
IVA	**Tümörün mesane ve/veya bağırsak mukozasına invazyonu**
IVB	**Uzak metastazlar (intraabdominal metastazlar ve/veya inguinal lenf nodları)**

2.3.3 Endometrium Kanserinde Prognostik Parametreler

Jinekolojik onkoloji grubu tarafından yapılan çalışmalar, endometrial karsinom prognostik parametrelerinin uterin ve ekstrauterin faktörler olarak iki grupta toplanabileceğini göstermiştir. Uterin faktörler (1) Histolojik tip, (2) Histolojik derece, (3) Myometriyal invazyon derinliği, (4) Vasküler invazyon, (5) Atipik hiperplazinin varlığı, (6) Servikal tutulum, (7) DNA ploidi ve S-fazı fraksiyonu ve (8) Hormon Reseptör şeklinde listelenilebilir. Ekstrauterin faktörler ise (1) Pozitif peritoneal sitoloji, (2) adneks tutulumu, (3) pelvik ve para-aortik lenf nodu metastazı ve (4) peritoneal metastazdır [33].

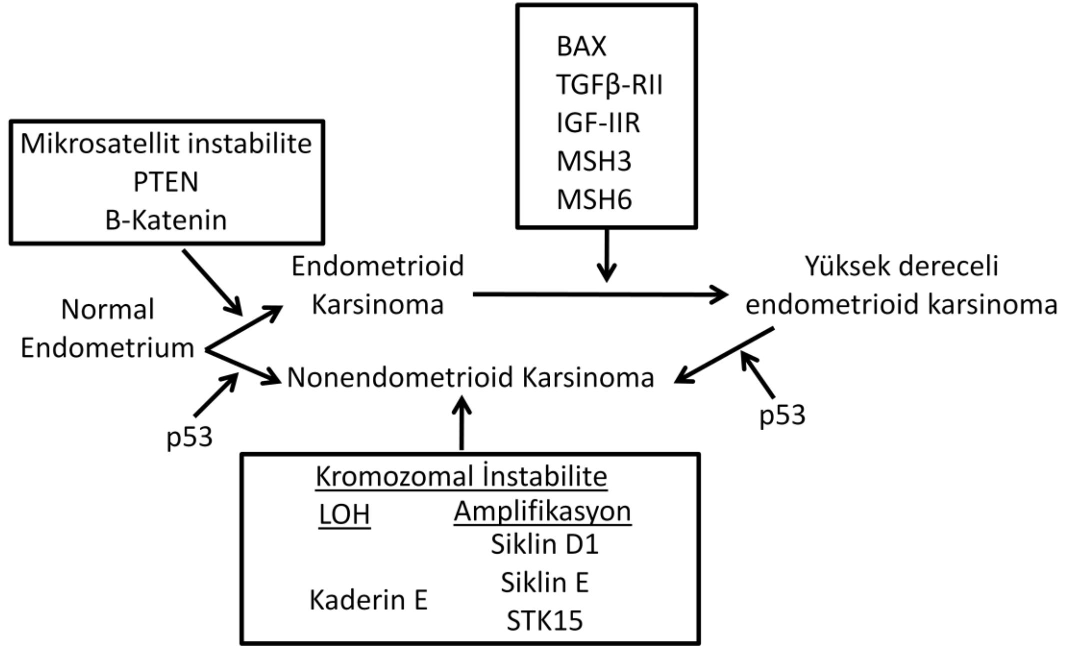
Endometrium kanserinde prognostik faktörlere yaş faktörünü de eklemek mümkündür. Endometrial kanserli genç kadınlarda yaşlı kadınlara göre daha iyi prognozla karşılaşılmaktadır. Yaşlı hastalarda karşılaşılan kötü prognoz daha çok grade 3 tümörlerin görülme sıklığındaki artıştan kaynaklanmaktadır. Aalder ve arkadaşlarının yapmış olduğu bir çalışmada 60 yaş altı kadın hastalarda mortalite ve rekürrens oranı % 6.1 iken 60 yaş üstünde bu oran % 12.3 olarak belirlenmiştir. Endometrium kanserinde hastalığın evresi sağ kalımı etkileyen en önemli faktördür. Kottmeimer ve arkadaşlarının 1971 FIGO evreleme sistemine göre yapmış oldukları çalışmada 5 yıllık sağkalım oranları Evre I %74.2, Evre II %57.4, Evre III %29.2 ve Evre IV % 9.6 olarak belirlenmiştir. Histolojik derece, hastalarda lenf nodu metastazı ve miyometrial invazyon varlığını yansıtması nedeni ile prognozla ilişkilidir. Derin myometrial invazyon da kötü sağkalım ile ilişkilidir. Boronow ve arkadaşları, endometrium ile sınırlı endometrial kanserli hastalarda hastalığın tekrarlama oranı % 8 iken derin myometrial invazyonlu hastalarda bu riskin arttığını göstermiştir. Myometrial invazyonsuz hastalarda 5 yıllık sağkalım oranı %80 iken derin myometrial invazyonda bu oran % 60'a düşmektedir [34].

2.3.4 Endometrium Kanserinde Genetik Değişimler

Normal bir hücreden kanser hücresinin gelişimi, normal endometriumun gelişiminde ve devamlılığında etkin olan genlerdeki mutasyonları kapsayan multistep bir olaydır (Şekil 2.3). Bu genler üç kategoride toplanmaktadır: proto-onkogenler, tümör baskılayıcı genler ve DNA tamir genleri. Bu moleküler değişimler endometrium kanserinin iki tipi arasında farklılıklar göstermektedir. Tip I Endometrial karsinomalarda mikrosatellit instabilitesi, PTEN, K-RAS, β-Katenin ve PIK3CA genlerinde değişimler söz konusudur. Tip II Endometrial karsinomada ise sıklıkla p53 mutasyonları, Her-2/neu amplifikasyonu ve birkaç kromozomda heterozigotluğun kaybı (LOH) gözlenmektedir [15, 35]. Bu genetik değişimler farklı bireylerde tek başına veya çeşitli kombinasyonlarda ortaya çıkabilmektedir [36]. Tip I ve II endometrial karsinomalardaki değişiklikler Tablo 2.7'de özetlenmiştir.

Tablo 2.7. Tip I ve Tip II Endometrial karsinomalarda genetik değişimler [37]

	Tip I	Tip II
PTEN inaktivasyonu	% 83	% 11
PIK3CA mutasyonu	% 26-36	% 5
KRAS mutasyonu	% 10-30	% 0-10
β-katenin/CTNNB1 mutasyonu	% 14-44	% 0-5
Mikrosatellit instabilitesi	% 20-45	% 0-11
p53 mutasyonu	% 10-20	% 90
HER2/neu amplifikasyonu	% 10-30	% 18-80
p16 inaktivasyonu	% 10	% 40-45
E-kaderin kaybı	% 10-20	% 60-90



Şekil 2.3 Endometrial karsinoma patogenezi [36].

K-ras: K-Ras geni GTPaz süperailesine ait bir proteini kodlamakta ve hücre yüzey reseptörleri ve nükleus arasındaki sinyal iletiminde rol oynamaktadır. Kodon 12, 13 veya 61'deki tek bir aminoasit değişimi aktive edici mutasyondan sorumludur [36]. K-ras proto-onkogeninin kodon 12 veya 13'ünde meydana gelen nokta mutasyonlarına endometrial karsinomaların % 10-37'sinde ve atipik hiperplazilerin % 6-16'sında rastlanırken, basit ve kompleks hiperplazide K-ras gen aktivasyonu söz konusu değildir. Tip I tümörlerde K-ras mutasyonu % 26 iken, bu oran tip II tümörlerde % 2'dir. Yapılan çalışmalara göre mutasyonların görülme sıklığı hiperplazilerin şiddetine göre değişmektedir, ki bu oranlar basit atipik hiperplazide % 10-14, kompleks atipik hiperplazide % 22 civarındadır [38]. K-Ras mutasyonları ile endometrioid karsinomalarda tümör evresi, histolojik derece, myometriyal invazyon derinliği ve klinik arasında bağlantı bulunamamıştır. Fakat bazı araştırmacılar, post-menopozomal endometrioid karsinomalı hastalarda K-Ras mutasyonları ile beraberinde bulunan endometrial hiperplazi, lenf nodu metastazları ve klinik arasında ilişkiler tanımlamışlardır [39-40].

HER-2/neu: HER-2/neu, transmembran tirozin kinaz kodlayan bir onkogendir. Bir büyüme faktör reseptörü olarak fonksiyon görür ve ErbB sinyalleşmesini koordine etmede önemli rol oynamaktadır. HER2/neu epidermal büyüme faktör reseptör (EGFR) ailesinin diğer üyeleri ile heterodimerize olarak iş görmektedir. HER2/neu aktivasyonu, mitojen-aktive protein kinaz ve PI3K hücre sinyalleşmesinde artışa yol açmakta bu da hücre proliferasyonunda artış ile sonuçlanmaktadır [41]. HER-2 / neu'nun ekspresyonunun artması endometrium kanserinde kötü prognostik faktördür [42]. HER2/neu onkogeninin amplifikasyonu veya overekspresyonu endometrial karsinomaların % 20-40'ında ortaya çıkmaktadır ve ileri evre, yüksek histolojik derece ve azalmış sağ kalım ile ilişkilendirilmektedir [43]. HER2/neu amplifikasyonu daha çok nonendometrioid karsinomada (Tip II) görülmektedir [44].

Mikrosatellit İnstabilitesi (MSI): DNA mikrosatellit genom boyunca yer alan 1-6 nükleotidlik basit dizi tekrarlarıdır. Mikrosatellit instabilitesi (MSI) mikrosatellit uzunluklarındaki değişimler olarak tanımlanmaktadır. Bu tip genetik instabilitenin DNA-mismatch tamir (MMR) genlerindeki bozukluktan kaynaklandığı düşünülmektedir [38]. MLH1, MLH3, PMS1, PMS2, MSH2, MSH3, MSH4, MSH5 ve MSH6 gibi genler insanda DNA mismatch tamir yolağında tanımlanmış olan genlerdir [45]. MMR genlerindeki mutasyonlar herediter nonpolipozis kolorektal kanser (HNPCC) patogenezinde ve Lynch's sendromunda önemli rol oynamaktadır. Endometrial karsinoma HNPCC'li hastalarda tanımlanmış en yaygın ikinci malignansi tipidir. Lynch's sendromu ile ilişkili endometrial karsinomaların %70'inde ve sporadik endometrial karsinomaların % 20-30'unda MSI tanımlanmıştır. Mikrosatellit instabiliteli sporadik kolonik, gastrik veya endometriyal karsinomlarda MMR genlerindeki mutasyonların sıklığı oldukça düşüktür ve bu durumda gen inaktivasyonlarında başka mekanizmaların rolü ileri sürülmektedir [36]. 1998 yılında MSI gösteren sporadik kanserlerde tümör baskılayıcı gen inaktivasyonunun mekanizması olarak MLH1 promotörünün hipermetilasyonu tanımlanmıştır [46]. MLH1 promotör hipermetilasyonu hiperplazilerde, özellikle atipik hiperplazilerde tanımlanmıştır. Bu bulgular, MLH1 hipermetilasyonunun endometrioid adenokarsinom patogenezi esnasında MSI fenotip gelişiminin erken basamağı olduğunu göstermektedir [47-48]. Sporadik endometrial karsinomlarda

MSI'ın klinikopatolojik etkisi tartışmalıdır. Histolojik derece veya sağkalım ile MSI arasındaki ilişki açık değildir. Bazı çalışmalarda yüksek histolojik derece ve kötü prognozla bir ilişkisinin var olduğunu öne sürülmekteyken, [49-50] son çalışmalara göre MSI, endometrial karsinomda iyi prognozu göstermektedir; örneğin kolorektal karsinomlarda evreden bağımsız olarak iyi prognozla ilişkilendirilmiştir [51].

PTEN: PTEN, 10q23.3 kromozom bölgesinde lokalize, tümör baskılayıcı bir gendir ve pek çok kanser tipinde bu bölgenin heterozigosite kaybına rastlanmaktadır. PTEN proteini protein tirozin fosfataz ve tensin homolojisine sahiptir. PTEN fosfataz aktivitesi ile PI3K ürünü olan fosfotidilinozitoltrifosfatların (PIP3) defosforilasyonunu yaparak PI3K/AKT yolağını baskılamaktadır. Bu nedenle PTEN aktivitesindeki bir azalma, sinyal transdüksiyon yolaklarının modülasyonu aracılığıyla hücre proliferasyonunda artışa neden olmaktadır [52]. Diğer taraftan PTEN aynı zamanda FAK (Fokal adhezyon kinaz) aracılığıyla hücre adezyonu ve migrasyonunda da işe karışmaktadır. PTEN, FAK defosforilasyonuna neden olarak, integrin aracılı hücre sinyalleşmesini inhibe etmekte ve hücre-ekstrasellüler matriks ilişkilerini bozmaktadır [53]. Endometrial kanserlerde PTEN mutasyonlarına oldukça sık rastlanılmaktadır. Özellikle, papillar seröz ve berrak hücreli nonendometrioid tip endometrial karsinomlarda bu oran dikkat çekicidir [54-55]. PTEN mutasyonlarının görülme sıklığı erken evre/iyi diferansiye endometrioid ve ileri evre/kötü diferansiye adenokarsinomlar arasında benzerlik göstermektedir. PTEN mutasyonları nükleer atipili ve atipisiz endometrial hiperplazilerin % 15-55'inde belirlenmiştir. Bu durum, PTEN fonksiyon kaybının endometrial karsinogenezin erken döneminde ortaya çıktığını göstermektedir [36, 56].

PIK3CA: PI3K'nın p110α katalitik altünitesi olan PIK3CA, 3q26.3 kromozom bölgesinde lokalizedir ve bu lokusun amplifikasyonu PI3K aktivitesini arttırmaktadır. PIK3CA insan kanser türlerinin yaklaşık % 15'inde aktive edilmiş veya mutasyona uğramıştır [57]. Endometrial karsinomlarda ise PIK3CA değişimlerine yaklaşık % 24-36 oranında rastlanmaktadır ve bu değişimlerin pek çoğu ekzon 20'deki yanlış anlamlı mutasyonlardır. Oda ve arkadaşları, yapmış oldukları çalışmada endometrial karsinomalı vakaların % 36'sında PIK3CA mutasyonları belirlerken, vakaların % 26'sında PTEN ve PIK3CA mutasyonlarının birlikte olduğunu göstermişlerdir. Aynı araştırmacılar PIK3CA mutasyonu ile klinikopatolojik parametreler arasında bir ilişki saptayamamışlardır ki, bu da PI3K yolağının tek başına aktivasyonunun endometrial karsinomlarda prognostik bir faktör olmadığını göstermektedir [58-59].

β-katenin/CTNNB1: β-katenin proteini 3p21 kromozom bölgesinde lokalize CTNNB1 geni tarafından kodlanmaktadır. β-katenin adheziv proteinleri grubuna girmekte ve bir hücre-hücre adezyon regülatörü olarak fonksiyon görmekle beraber aynı zamanda Wnt sinyal yolağının bir bileşeni olarak hareket etmektedir. Hem APC (adenomatous polyposis coli) hem de E-kaderin'in fonksiyonel aktivite gösterebilmesi için gereklidir. Normal doku yapısının korunması ve hücre farklılaşmasında temel olan E-kaderin-katenin ünitesinin bir komponentini oluşturmakla beraber, Wnt sinyal yolağında da önemli bir rol oynamaktadır. CTNNB1 geninin 3 nolu ekzonunda meydana gelen mutasyonlar β-katenin proteinin stabilizasyonuna ve nüklear birikimine yol açmaktadır. β-katenin nüklear birikimi

gösteren endometrial karsinomlar analiz edildiğinde birikimin endometrioid lezyonlarda (% 31-47) nonendometrioid histolojilere nazaran (% 0-3) önemli derecede daha yaygın olduğu gösterilmiştir. APC/β-katenin/Tcf yolağının aktivasyonu bazı kanser tiplerinde görülmekte, özellikle endometrioid endometrial karsinomlarda daha sık rastlanmaktadır. Endometrioid endometrial karsinomlarda CTNNB1 mutasyonlarının görülme sıklığı % 14-44 olarak rapor edilmiştir. CTNNB1 mutasyonu bulunan endometrioid endometrial karsinomalar karakteristik olarak iyi prognozlu erken evre tümörler ile ilişkilidir [36-37, 60-61].

p53: p53 tümör baskılayıcı gen 17p13.1 kromozom bölgesinde lokalizedir. p53 mutasyonları nonendometrioid endometrial karsinomaların % 90'ında gözlenirken endometrioid endometrial karsinomaların (özellikle grade 3) sadece % 10-20'sinde gözlenmektedir. Bu bulgu, nonendometrioid karsinomlarda değil ama, endometrioid endometrial karsinomlarda hastalık progresyonunu etkileyebileceğini desteklemektedir. p53'ün overekspresyonu yüksek evre, agresif hücre tipi (seröz), yüksek histolojik grade ve derin myometriyal invazyon ile ilişkili bulunmuştur. Azalmış sağkalım ile de kuvvetli bir ilişki bulunmaktadır [62-63].

E-Kaderin: Kaderinler hücreler arasında sıkı bağlantı için temel adezyon moleküllerinin bir ailesidir. E-kaderin 16q22.1 kromozom bölgesinde lokalize CDH1 geni tarafından kodlanmaktadır. E-kaderin ekspresyonundaki azalma endometrial karsinomda sıktır ve ekspresyondaki azalma LOH, mutasyon veya promotor hipermetilasyonu kaynaklı olabilir. 16q22.1'de LOH nonendometrioid endometrial karsinomaların % 60'ında gözlenirken, endometrioid endometrial karsinomaların sadece % 22'sinde gözlenmektedir. Endometrial karsinomada, E-kaderin ekspresyonunun kısmi ya da tam kaybı agresif durum ile ilişkilidir [36].

p16: p16 hücre döngüsünün düzenlenmesinde önemli bir rol oynamaktadır. p16 tümör baskılayıcı geni 9p21 kromozom bölgesinde lokalizedir. p16 inaktivasyonu kontrolsüz hücre büyümesine yol açmaktadır. p16 inaktivasyonu nonendometrioid endometrial karsinomada (% 40-45) endometrioid endometrial karsinomaya (% 10) nazaran daha sık gözlenmektedir. p16 ekpresyon kaybı KRAS ve p53 mutasyonları ile koreledir ve ileri evre, yüksek derece ve kötü sağkalım ile ilişkilidir [64].

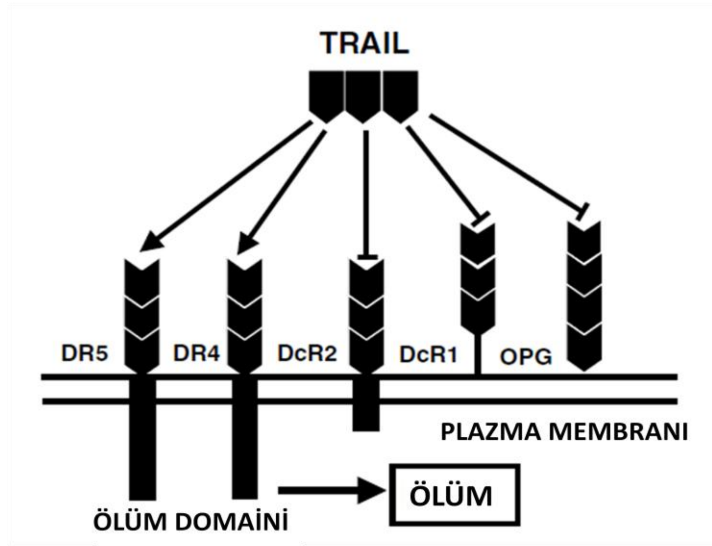
2.4. TRAIL

1995 ve 1996 yıllarında, iki bağımsız grup tarafından direkt olarak apoptotik sinyali başlatabilme yeteneğinde olan yeni bir protein keşfedilmiştir. Bu protein ile TNF ve Fas Ligand arasındaki yüksek homoloji nedeniyle bir grup tarafından TNF-related apoptosis-inducing ligand (TRAIL), diğer grup tarafından ise Apo2L olarak adlandırılmıştır [65-66].

TRAIL geni insanda 3q26 kromozom bölgesinde lokalizedir. 20 kb uzunluğunda olup, 5 ekzondan oluşmaktadır [67-68]. TNF ailesi üyesi olan TRAIL bir tip II transmembran proteinidir. 281 aminoasitten oluşan TRAIL proteini kısa bir N-terminal sitoplazmik domainine ve uzun bir C-terminal ekstraselüler reseptör bağlanma domainine sahiptir [69].

TRAIL'ın en önemli özelliği tümörojenik veya transforme olmuş hücrelerde seçici olarak apoptozu indüklemesi, buna karşın normal hücrelerde apoptozisi indükleyememesidir. Bu özelliği, TRAIL'ın kanser tedavisinde uygulanmasına olanak sağlamaktadır [70].

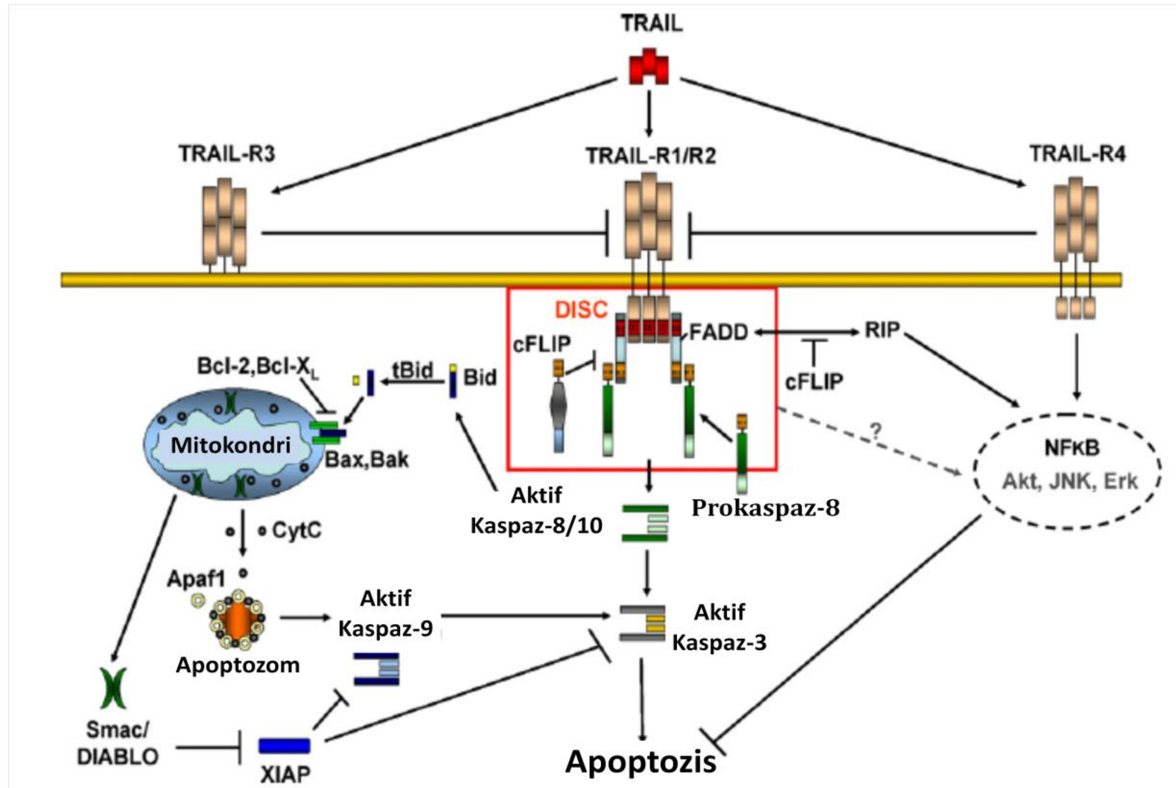
TRAIL, etkisini hücre yüzeyinde bulunan beş farklı TRAIL reseptörü aracılığıyla gerçekleştirir (Şekil 2.4). Bunlar TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/DcR1, TRAIL-R4/DcR2 ve Osteoprotegrindir [71]. TRAIL-R1 (DR4, TNFRSF10a) ve TRAIL-R2 (DR5, TNFRSF10b) sisteince zengin iki ekstraselüler ligand bağlanma domaini ve TRAIL bağlandıktan sonra ekstrinsik apoptotik yolağın aktivasyonu için gerekli ölüm domainini içermektedirler. Bu nedenle bu iki reseptör ölüm reseptörleri olarak adlandırılmaktadırlar. TRAIL-R3 (DcR1, TNFRSF10c), TRAIL-R4 (DcR2, TNFRSF10d) ve osteoprotegrin (OPG, TNFRSF11b) ölüm domaininden yoksun olmalarından dolayı, gelen sinyali aktaramazlar ve yalancı reseptörler olarak tanımlanırlar [72].



Şekil 2.4. TRAIL ve reseptörleri [73].

Memeli hücrelerinde apoptotik sinyali başlatabilen iç ve dış yolak olmak üzere iki ana sinyal yol tanımlanmıştır. Dış sinyal yolağı TRAIL homotrimerlerinin hedef hücre yüzeyinde bulunan DR4 veya DR5 ölüm reseptörlerinden birine bağlanması ve reseptör trimerizasyonunu indüklemesi ile başlar. Ölüm domainlerinin trimerizasyonu FADD (Fas associated death domain) olarak adlandırılan adaptör proteinin çağrılması ile sonuçlanır. Sonuç olarak, ölümü-indükleyici sinyal kompleksi (DISC) olarak adlandırılan bir reseptör kompleksi oluşur [71, 74].

FADD, biri ölüm domaini (DD) ve diğeri ölüm effektör domaini (DED) olmak üzere iki domainden oluşmaktadır. FADD, ölüm domaini aracılığı ile reseptöre bağlanırken, ölüm effektör domaini ile de pro-kaspaz 8 veya pro-kaspaz-10'u aktive eder. Kaspaz 8 ve kaspaz 10 ise, hücrenin apoptotik ölümünü sağlayan efektör kaspazları, yani kaspaz 3, 6 ve 7'yi aktive eder. İç apoptotik yolak ise, TRAIL uyarımı sonrasında aktif kaspazların bir pro-apoptotik Bcl-2 ailesi üyesi olan Bid'i keserek aktive etmesiyle başlar. Aktive olan Bid molekülü, Bax ve Bak molekülleri ile etkileşime girerek mitokondriden sitokrom-c ve Smac/Diablo salınımını sağlar. Sitokrom-c, adaptör Apaf-1'e bağlanarak apoptozom kompleksinin oluşumunu tetikler ve kaspaz 9 aktivasyonunu sağlar. Bu aktivasyon diğer efektör kaspazlar olan 3, 6 ve 7'nin aktivasyonuna neden olur. Effektör kaspazların aktivasyonu ile hücre yine geri dönüşümsüz olarak apoptoza gider. Smac/Diablo ise, Inhibitor of Apoptosis Protein (IAP) moleküllerine bağlanır. Bu bağlanma, IAP'lerin kaspaz 3'e bağlanmasını engeller. Dolayısıyla kaspaz 3'ün aktif durumunun devamı sağlanmış olur [71, 74] (Şekil 2.5).



Şekil 2.5. TRAIL sinyal yolağı [69].

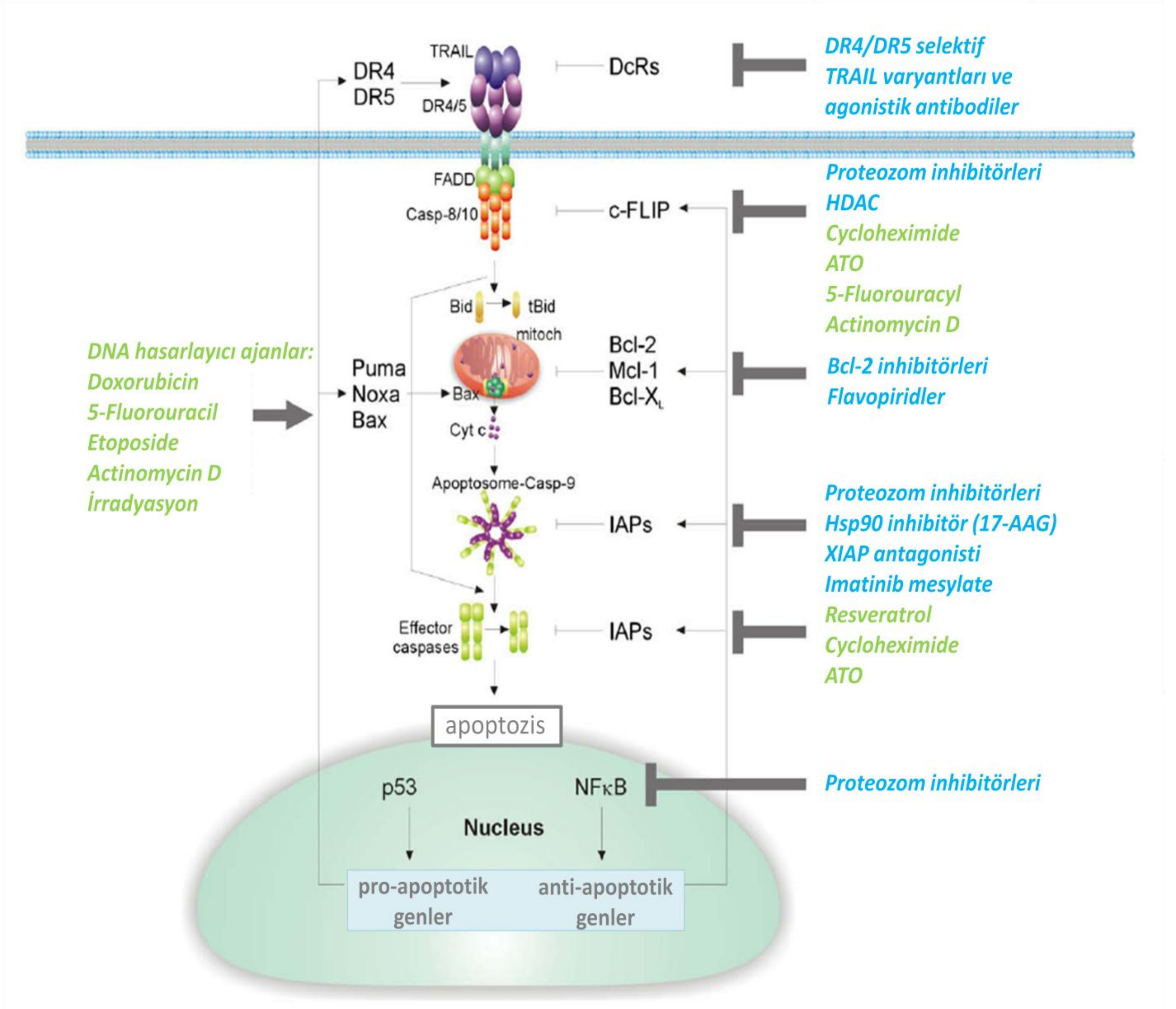
2.4.1. TRAIL'ın Fizyolojik Rolü

Yapılan çalışmalarda TRAIL mRNA'sı PBMC, dalak, timus, prostat, ovaryum, ince bağırsak, kolon ve plasentayı da içeren pek çok dokuda eksprese edilirken, karaciğer, testis veya beyinde eksprese edilmemektedir [66].

Pek çok dokuda yaygın olarak sentez edilen TRAIL'ın, tümöre karşı fonksiyon gören bir ajan olarak aktivitesi kabul görmüş olsa da, normal fizyolojik şartlardaki fonksiyonu tam olarak açığa çıkarılamamıştır [75]. TRAIL'in onkojenik transformasyona uğramış veya viral enfeksiyon geçirmiş hücrelere karşı immün cevapta önemli bir oyuncu olabileceği düşünülmektedir. Sitotoksik CD4+ T lenfositleri ve NK hücreleri ile hedef hücre eliminasyon mekanizmasında rol aldığına dair bulgular mevcuttur [74]. TRAIL knockout farelerle yapılan çalışmalar, NK hücreler tarafından sağlanan antitümör etkide özellikle tümör oluşumu ve metastaza karşı konakçı savunmasında TRAIL'ın rolünü doğrulamaktadır [76]. Ayrıca, TRAIL'in, viral enfeksiyona karsı IFN (Interferon)-bağımlı konakçı savunmasının erken evresinde rol oynayabileceği düşünülmektedir [74, 77].

2.4.2. TRAIL Dirençlilik Mekanizmaları

İnsan ve fare tümör hücre hatlarının pek çoğu TRAIL tarafından indüklenen apoptozise karşı duyarlı olsa da, TRAIL'e dirençlilik gösteren birçok primer tümör de bulunmaktadır. Ancak normal hücreler ve belirli tümör hücrelerindeki dirençliliğin nedeni tam olarak açıklanamamıştır. Bu dirençlilikle ilişkili potansiyel mekanizmalar düşünülmektedir. TRAIL aracılı apoptozis dirençlilik mekanizmaları ve potansiyel terapötik duyarlılaştırma yöntemleri Şekil 2.6'da gösterilmiştir.



Şekil 2.6. TRAIL aracılı apoptozis dirençlilik mekanizmaları ve potansiyel terapötik duyarlılaştırma yöntemleri [78].

TRAIL reseptör mRNA'larının çeşitli normal hücre ve dokularda sürekli olarak sentezlendiği gösterilmiştir ve dört TRAIL reseptörü de farklı affiniteler ile TRAIL'a bağlanmaktadır [79-80]. Bu nedenle, TRAIL dirençliliğini açıklayabilecek faktörlerden birinin yalancı reseptörler TRAIL-R3 ve TRAIL-R4 ile FADD içeren TRAIL-R1 ve TRAIL-R2 arasındaki yarış sonucunda TRAIL sinyalleşmesinin negatif regülasyonu olabileceği düşünülmekteydi [81-82]. Bu hipotez, TRAIL-R3 veya TRAIL-R4 overekprese eden TRAIL duyarlı hücreler kullanılarak yapılan deneylerde TRAIL-aracılı apotozisin inhibe edilmesi ile desteklenmiştir. Birçok gastrointestinal tümör örneğinde de, TRAIL-R3 aşırı ekspresyonu sonrasında TRAIL'e dirençliliğin ortaya çıktığının gösterilmesi sonrasında TRAIL-R3 veya TRAIL-R4'ün koruyucu rolü daha yaygın bir şekilde kabul görmüştür [83]. Buna ek olarak, grubumuz tarafından daha önce gerçekleştirilmiş olan bir çalışmada da TRAIL-R4 ekspresyon seviyesi ile insan meme tümör hücre hatlarında TRAIL dirençiliği arasında ilişki bulunduğu saptanmıştır [84]. siRNA kullanılarak özellikle TRAIL-R4'ün hedef alındığı akciğer ve prostat tümör hücrelerinde yapılan çalışmalarda ise TRAIL'e karşı duyarlılık sağlandığı gösterilmiştir [85-86]. Diğer taraftan, insan melanoma hücre hatlarında yapılan bir çalışmada ise yalancı reseptör ekpresyonu ve TRAIL dirençliliği arasında korelasyon bulunamazken, hücre yüzeyi TRAIL-R1 veTRAIL-R2 ekpresyonu ile apoptozis arasında korelasyon gösterilmiştir [87].

TRAIL reseptör mutasyonlarının da bazı tümörlerde TRAIL reseptör aracılı sinyalleşmeyi engellediği düşünülmektedir. Baş-boyun kanseri, akciğer kanseri, meme kanseri, non-hodgkin's lenfoma, kolorektal kanser, gastrik kanser ve hepatoselüler karsinomda yapılan çalışmalarda TRAIL-R2 geninde özellikle ölüm domeini içinde lokalize mutasyonlar saptanmıştır [88-94].

TRAIL-indüklü apoptozise karşı tümör hücrelerinde duyarlılık farklarını açıklamaya yönelik bir diğer yaklaşım ise hücre döngüsü progresyonu ile duyarlılık arasındaki ilişkidir. Jin ve arkadaşları tarafından SW480 kolon kanser ve H460 akciğer kanser hücre hatlarında yapılmış bir çalışmada bu hücreler G0/G1 fazında durdurulduğu zaman TRAIL-indüklü apoptozise duyarlılığın diğer fazlarda durdurulmuş aynı hücrelere göre arttığı gösterilmiştir. Bu mekanizmanın altında yatan nedenin, farklı hücre fazlarında anti-apoptotik protein düzeylerinin farklı olması olabileceği düşünülmektedir [95]. Dirençlilik ile ilişkili bir başka hipotezde ise TRAIL'e dirençli hücrelerde pro-apoptotik ve anti-apoptotik moleküllerin ekpresyonlarının arttığı öne sürülmektedir. TRAIL sinyal yolağında DISC oluşumu esnasında, cFLIP FADD'a bağlanmada yarışarak kaspaz aktivasyonunu inhibe etmektedir. Nitekim cFLIP'in baskılanmasının bazı kanser hücrelerini TRAIL-indüklü apoptozise karşı duyarlı hale getirdiği yapılan çalışmalarda gösterilmiştir [96]. Tümör hücrelerinde apoptozis inhibitör proteinlerin (IAP) yüksek düzeyde varlığı da kaspaz 3, 7 ve 9'u direkt olarak inhibe ederek TRAIL dirençliliğine yol açmaktadır. Bcl-2 ve Bcl-xL gibi Bcl-2 ailesinin anti-apoptotik üyelerinin aşırı ekspresyonunun da tip II hücrelerde TRAIL dirençliğine neden olduğu gösterilmiştir [78].

2.4.3. Kanserlerde TRAIL/TRAIL Reseptör Ekspresyon Profili

Günümüzde TRAIL reseptör ekspresyonlarının prognostik önemi, hastaların tedavisi açısından potansiyel etkilere sahip olması nedeni ile önemli bir araştırma konusu haline gelmiştir. Bu nedenle, immunohistokimya çalışmaları farklı histolojik derecelerdeki servikal, ovaryum ve kolon tümörlerinde TRAIL reseptör ekspresyon profillerinin ve TRAIL lokalizasyonunun normal dokular ile karşılaştırılmasında kullanılmaktadır. Ovaryum, kolon ve servikal olmak üzere değerlendirilen üç kanser tipinde de, TRAIL ekspresyon düzeyinin yüksek dereceli lezyonlarda artış gösterdiği, buna karşın DR4 ve DR5 reseptör ekspresyonunun pre-malignant hücreler, benign tümörler ve normal epitelyum ile karşılaştırıldığında tümör hücrelerinin büyük bir yüzdesinde oldukça yüksek oranda belirlenmiştir [97-99]. Bir başka çalışmada ise rezeksiyonu takiben adjuvan tedavi alan 376 evre III kolon kanserli hastada immunohistokimya ile TRAIL, DR4 ve DR5 ekspresyonları değerlendirilmiştir. Bu tümör örneklerinde % 83 oranında yüksek düzeyde TRAIL, % 92 DR4 ve % 87 DR5 ekspresyonu belirlenmiştir. Yüksek DR4 ekspresyonu kısa süreli sağkalım ve kısalmış nüks zamanı ile ilişkili bulunmuştur [100][100][100][100]. Benzer şekilde 655 erken evre meme kanserli hastalardan alınan örneklerde yapılan doku mikroarray analizlerinde DR5 ekspresyonu ile hastalarda azalmış sağkalım oranı ve yüksek lenf nodu yayılımı arasında bir ilişki olduğu saptanmıştır [101]. Kötü prognozlu tümörlerde artmış DR5 ölüm reseptör ekspresyonu, bu tümörlerin TRAIL-aracılı immün kontrolden kaçabilmesine neden olarak daha agresif bir progresyona yol açıyor olabilir. Nitekim TRAIL dirençli tümörlerin lenf nodu metastaz oranlarında da artış belirlenmiştir [102]. Yine de hastalığın agresifliği ve tümör progresyonunda TRAIL duyarlılığının rolünü açıklamak için TRAIL reseptör ekspresyonu dışında ne gibi faktörlerin rol aldığının belirlenmesine yönelik daha fazla çalışma yapılması gerekmektedir.

Yapılan çalışmalarda TRAIL ligand ve reseptör ekspresyon profillerinin kanser tipleri arasında farklılık gösterdiği saptanmıştır. İleri evre prostat kanserli hastalarda DcR2 seviyesi erken evrelere oranla daha yüksek olarak belirlenmekle beraber, DcR2 sentez düzeyi ile PSA ve Gleason skorlama gibi prostat kanserinin takibinde kullanılan belirteçler arasında bir ilişki saptanmış ve DcR2 sentez düzeyindeki artış azalmış sağkalım ile ilişkili bulunmuştur [103-104]. Meme kanserinde ise DR4 ekspresyonu ile tümör derecesi arasında pozitif bir korelasyonunun varlığı gösterilmiştir [105]. Tüm bu sonuçlar TRAIL ligand ve reseptör ekspresyon profillerinin kanser prognozunu belirlemede bir belirteç olarak kullanılabileceğini işaret etmektedir.

MATERYAL VE METODLAR

2000-2009 yılları arasında Akdeniz Üniversitesi Tıp Fakültesi Kadın Hastalıkları ve Doğum Anabilim Dalı tarafından uygulanan cerrahi sonrası Akdeniz Üniversitesi Tıp Fakültesi Patoloji Anabilim Dalı arşivinde depolanmış formalin-fikse, parafine gömülü 100 Endometrioid Tip Adenokarsinoma, 27 Endometrial Hiperplazi ve 18 Normal Endometrium dokusu bu çalışmaya dahil edilmiştir.

3.1. İmmunohistokimyasal Boyama Yöntemi

Dokularda TRAIL, TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (DcR1) ve TRAIL-R4 (DcR2) varlığını göstermek amacıyla immunohistokimya yöntemi kullanılmıştır. Kullanılan solüsyonlar ve yöntem aşağıda ayrıntılı olarak verilmiştir.

3.1.1. Kullanılan Solüsyonların Hazırlanması

1X PBS (Phosphate buffered saline) Tamponu:

7.2 gr. $Na_2HP0_4.12H_20$ (Merck)
0.8 gr. KH_2P0_4 (Merck)
16 gr. NaCl (Merck)

Yukarıdaki kimyasallar 2 litre bidistile su içerisinde çözülerek 2N NaOH kullanılarak pH'ı 7.4 olacak şekilde ayarlandı. Hazırlanan solüsyon +4°C'de saklandı.

Sitrik Asit Tamponu:

100 ml Citrate buffer (10X) (Lab Vision Corporation) ve 900 ml distile su karıştırılarak hazırlandı.

%3'lük H_20_2 Solüsyonu:

Peroxide Block (ScyTek Laboratories) kullanıma hazır olarak alındı ve +4°C'de saklandı.

Bloklama Solusyonu:

Ultra V Block (Lab Vision Corporation) kullanıma hazır olarak alındı ve +4°C'de saklandı.

Primer Antikor Dilüsyon Solüsyonu:

Large Volume UltrAb Diluent (Lab Vision Corporation) kullanıma hazır olarak alındı ve +4°C'de saklandı.

Sekonder Antikor:

LSAB+System HRP (Biotinylated Link Universal, Streptavidin-HRP) (DAKO) kullanıma hazır olarak alındı ve +4°C'de saklandı.

DAB Solüsyonu:
Large Volume DAB substrate system (Lab Vision Corporation) satın alınarak kullanımdan hemen önce 1ml DAB substrat tamponu içerisine 1 damla (~40µl) DAB kromojen karıştırılarak hazırlandı.

3.1.2. İmmunohistokimya Boyamasının Optimizasyonu:

Hastalara ait preparatlarda hedef antijene spesifik antikorlar ile immunohistokimyasal boyamaya geçmeden önce pozitif kontrol boyamaları yapılarak gerek antijen retrieval için tercih edilecek tampon çözeltinin (EDTA veya Sitrat tamponu), gerekse tüm örneklerin boyamasında kullanılacak antikorların dilüsyonlarının optimizasyonları yapıldı. Pozitif kontrol olarak, kullanılacak olan TRAIL, TRAIL-R1, TRAIL-R2, TRAIL-R3 ve TRAIL-R4 antikorları ile pozitif reaksiyon verdiği bilinen lenf dokuları kullanıldı. Lenf dokusundan hazırlanan kesitlerde her bir antikor için 1/50, 1/100, 1/200 ve 1/300 dilüsyonlarda boyamalar yapıldı. Işık mikroskobunda yapılan analizlerde optimum antikor dilüsyonlarına karar verildi. Kullanılan antikorlar ve optimum dilüsyonları Tablo 3.1'de özetlenmiştir.

Tablo 3.1. Primer antikor listesi ve optimum dilüsyon oranları

Primer Antikor	**Dilüsyon Oranı**
TRAIL (ALX-804-326-C100, Alexis)	1/50
TRAIL-R1/DR4 (ALX-804-297-C100, Alexis)	1/50
TRAIL-R2/DR5 (ALX-210-743-C200, Alexis)	1/200
TRAIL-R3/DcR1 (ALX-210-744-C200, Alexis)	1/300
TRAIL-R4/DcR2 (ALX-804-299-C100, Alexis)	1/100

3.1.3. İmmunohistokimyasal Boyama İşlemleri:

1. Formalin fiksasyonlu parafine gömülü dokulardan alınan 5 mikronluk kesitler dokuların açılması için önce 40-45^{0}C sıcaklıktaki su banyosuna tabi tutulup ardından pozitif şarjlı lamlara alındı.

2. Kesitler 56 ^{0}C etüvde bir gece veya 60 ^{0}C etüvde 1 saat bekletildi.

3. Etüvden çıkarılan kesitler her biri 10'ar dakika olmak üzere Ksilol ile iki kez muamele edildi.

4. %100, %90, %80 ve %70'lik alkollerde 5'er dakika bekletildikten sonra distile sudan geçirilerek deparafinizasyon işlemi tamamlandı.

Sonuçta, formalin ile fikse edildikten sonra parafinle dolgu desteği sağlanmış dokulardan alınan kesitlerde doku örnekleri mevcut parafinin uzaklaştırılmasıyla immunohistokimyasal boyamaya hazır hale getirildi.

5. Deparafinizasyonu takiben formalin fiksasyonu sırasında dokuda oluşan kovalent bağların hidroliz edilerek protein moleküllerinin renatürasyonu ve böylece maskelenmiş antijenlerin açığa çıkarılması için antijen retrieval olarak adlandırılan işleme geçildi. Sepetlere yerleştirilen preparatlar PT Module (Lab Vision Corporation) sistemine konarak 98 ^{0}C'de 40 dakika sitrat tamponu içerisinde tutularak antijen retrieval işlemi tamamlandı.

6. PT modülden çıkarılan preparatlar oda sıcaklığında 20 dakika soğumaya bırakıldı.
7. Preparatlar distile su ve ardından PBS ile 2-3 dakika yıkanarak doku çevresi, dokuların kurumasına izin verilmeden silindi.

8. Dokuların çevresi hidrofobik bir kalemle (PAP-pen) çizilerek solüsyonların doku üzerinde kalması için sınırlandırıldı.

9. Preparatlar, %3 H2O2 solüsyonu ile 10 dakika oda sıcaklığında muamele edilerek endojen peroksidaz aktivitesi bloke edildi.

10. Preparatlar distile su ve ardından PBS ile 2-3 dakika yıkandı.

11. Ultra V Block solüsyonunda 10 dakika boyunca oda sıcaklığında bekletilerek özgül olmayan bağlanımların bloke edilmesi sağlandı.

12. Bloklama solüsyonu uzaklaştırılarak, örnekler üzerine uygun dilüsyonlarda hazırlanmış primer antikorlar eklendi ve +4^{0}C'lik nemli ortamda gece boyu inkübe edildi.

13. PBS ve distile su ile yıkama yapıldı.

14. Biotinlenmiş sekonder antikor ile muamele edildi (Bu işlem 2 aşamalıdır: 1. aşama; Anti-polyvalent ile 1 saat oda ısısında bekletme, ara yıkama; 2. aşama; Streptavidin Peroksidaz ile 15 dak. oda ısısında bekletme)

15. PBS ve distile su ile yıkama yapıldı.

16. DAB (Diaminobenzidine) solüsyonu damlatılıp, ortaya çıkan kromojenik reaksiyon ışık mikroskobu altında takip edilerek boyanma şiddeti belirlendi.

17. İnkübasyon süresi sonunda preparatların distile su içerisine alınması ile kromojenik reaksiyon durduruldu.

18. Mayer hematoksilen solüsyonu ile zıt boyama işlemi yapıldı ve çeşme suyu altında preparatlar yıkandı.

19. Boyanan preparatlar %70, %80, %90 ve %100'lik alkollerde 5'er dakika ve Ksilol'de iki kez 5er dakika bekletildi.
20. Preparatlar Entellan damlatılarak lamelle kapatıldı.

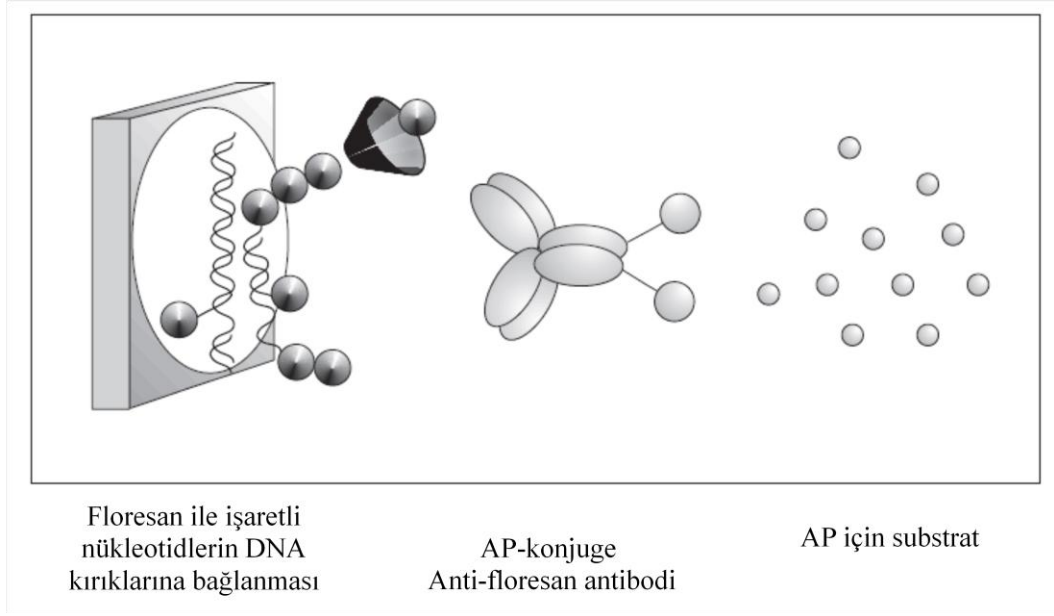
21. Işık mikroskobunda analiz aşamasına geçildi.

3.1.4. İmmunohistokimyasal Değerlendirme:

Boyaması yapılan preparatlarda ışık mikroskobunda doku boyanmaları değerlendirilirken, pozitif boyanan tümör hücrelerinin yaygınlığına ve boyanma yoğunluğuna göre olgular skorlandı. Tümör hücrelerinde boyanma yoğunluğu, boyanma yok (0), zayıf boyanma (+1), orta şiddette boyanma (+2) ve kuvvetli boyanma (+3) olarak değerlendirildi. Boyanma yaygınlığına göre sınıflandırma % 0-10 (0), % 10-40 (+1), % 40-70 (+2), % 70-100 (+3) olarak kabul edildi. Final skor, boyanma yoğunluğu ve boyanma yaygınlığı değerlerinin toplanması ile elde edildi.

3.2. TUNEL ((Terminal deoxynucleotidyl transferase (Tdt)-mediated dUTP-biotin nick end-labeling) Yöntemi:

Apoptotik sinyaller, DNA üzerinde kırıklar oluşturmaktadır. TUNEL adı verilen yöntemde, floresan işaretlenmiş deoksinukleotidler erken apoptoz esnasında açığa çıkan DNA parçacıklarının serbest 3'-OH uçlarına terminal deoksinükleotidil transferaz (TdT) aracılığı ile eklenir. Bu aşamada DNA kırıkları floresan mikroskopta değerlendirilebilir. Eğer değerlendirme ışık mikroskobunda yapılacaksa bu işaretlenmiş nükleotidler, alkalin fosfataz konjuge edilmiş anti-floresan antikorların da eklenmesi ile tespit edilir. Fast Red boya, işaretlenmiş örnek ile tepkimeye girerek DNA kırığı bölgesinde çözünmeyen bir substrat oluşturur. Hematoksilen ile ters boyama yapılarak işlem sonlandırılır. Bu işaretlenme sonucunda apoptozise yönelmiş hücreler görünebilir hale getirilir ve mikroskopla gözlenir (Şekil 3.1).



Şekil 3.1. TUNEL yöntemi prensibi [106].

3.2.1. Kullanılan Solüsyonların Hazırlanması

TUNEL Assay Kit:
TUNEL yönteminde, ticari olarak hazırlanmış TUNEL Assay Kit (In Situ Cell Death Detection Kit-AP, Roche Applied Science) kullanıldı. Uygulama Kit kılavuzunda belirtildiği şekilde yapıldı. Kit, enzim solüsyonu, işaretleme solüsyonu ve Converter-AP'den oluşmaktadır.

0.1 M Tris-HCl solüsyonu:
1.2 gr Tris (Amresco) tartılarak 100 ml distile suda çözdürüldü. HCl ile pH:8.2'ye ayarlandı. Hazırlanan solüsyon +4 ^{0}C'de saklandı.

10 mM Tris-HCl solüsyonu:
0.1 gr Tris (Amresco) tartılarak 100 ml distile suda çözdürüldü. HCl ile pH:7,4-8 olacak şekilde ayarlandı. Hazırlanan solüsyon +4 ^{0}C'de saklandı.

Proteinaz K solüsyonu:
Proteinaz K (Sigma-Aldrich) son konsantrasyonu 20µg/ml ve pH. 7,4-8 olacak şekilde 10 mM Tris-HCl ile hazırlandı.

TUNEL seyreltim tamponu:
TUNEL Dilution Buffer (Roche Applied Science) kullanıma hazır olarak alındı.

Fast Red Boya Solüsyonu:
Kullanımından hemen önce 2 ml 0.1 M Tris-HCl (pH: 8.2) içerisinde 1 tablet Fast Red (Roche Applied Science) çözdürülerek hazırlandı.

Kapatma Solüsyonu:
Kaisers Glycerin Gelatine (Merck) 60 ^{0}C'de eritilerek, çalışmaya başlamadan 10 dakika önce oda sıcaklığına çıkarıldı.

3.2.2. TUNEL Metodu:

1. Kesitler 60 ^{0}C etüvde 1 saat bekletildi.

2. Etüvden çıkarılan kesitler her biri 10'ar dakika olmak üzere iki kez Ksilen ile muamele edildi.

3. %100, %90, %80 ve %70'lik alkollerde 5'er dakika bekletildikten sonra PBS ve distile sudan geçirilerek dokuların etrafı dokular kurutulmadan silindi.

4. Dokuların çevresi hidrofobik bir kalemle (PAP-pen) çizilerek solüsyonların doku üzerinde kalması için sınırlandırıldı.

5. Dokuların üzeri kapanacak şekilde Proteinaz K çalışma solüsyonu (20µg/ml) damlatılarak preaparatlar 37 ^{0}C' de 20 dakika inkübe edildi.

6. PBS solüsyonunda 2 kez 5'er dakika yıkama yapıldı.

7. Bu aşamada TUNEL solüsyonu hazırlanarak preparatlara damlatıldı ve 37°C'de, 1 saat, nemli ve karanlık ortamda inkübe edildi. (TUNEL solüsyonu: 50µl enzim solüsyonu + 450 µl işaretleme solüsyonu (label solution) + 500 µl tunel dilution buffer karıştırılarak hazırlandı.) Negatif kontrole ise sadece 100 µl işaretleme solüsyonu + 100 µl TUNEL seyreltim tamponu karıştırılarak eklendi.

8. PBS solüsyonunda 2 kez 5'er dakika yıkama yapıldı.

9. Dokular üzerine 50 µl Converter-AP eklenerek 37°C'lik nemli ve karanlık ortamda 30 dakika inkübe edildi.

10. PBS solüsyonunda 2 kez 5'er dakika yıkama yapıldı.

11. Hazırlanan Fast-Red boya dokular üzerine damlatılarak oda sıcaklığında, 10 dakika, nemli ve karanlık ortamda inkübe edildi. Pozitif kontrolde sinyaller kontrol edilerek, renk değişimi görülür görülmez işlem durduruldu.

12. PBS solüsyonunda 2 kez 5'er dakika yıkama yapıldı.

13. Hemotoksilen ile zıt boyama yapıldı.

14. Kaisers Glycerin Gelatine preparatlar üzerinde damlatılarak lamelle kapatıldı.

3.2.3. TUNEL Analizi:

Apoptotik indeks oranı, hazırlanan her bir preparatta ışık mikroskobunda rastgele odaklanan 10 ayrı tümör bölgesinin değerlendirilmesi ile elde edildi. Her bir tümör bölgesinde sayılan apoptotik hücre sayısının toplam hücre sayısına bölünmesiyle ortaya çıkan oranların ortalaması alınıp bu oran 100 ile çarpılarak % Aİ (Apoptotik İndeks) değeri elde edildi.

3.3. İstatiksel Analiz

İstatistiksel analizler SPSS 16.0 paket programı kullanılarak yapıldı. Değerlendirme esnasında istatistiksel anlamlılık sınırı $p<0.05$ olarak kabul edildi. İstatistiksel analizde normalite testleri (Kolmogorov-smirnov, Shapiro-Wilk), Friedman testi, Wilcoxon Signed Rank test, Mann Whitney, Kruskal Wallis ve Spearman's rho korelasyon testi kullanıldı. Grafiklerin hazırlanmasında ise GraphPad Prism 5 paket programı kullanıldı.

BULGULAR

2000-2009 yılları arasında Akdeniz Üniversitesi Tıp Fakültesi Kadın Hastalıkları ve Doğum Anabilim Dalı tarafından opere edilerek Patoloji Anabilim Dalı arşivinde toplanan 100 endometrial karsinomlu, 27 endometrial hiperplazili olguya ait doku ve kontrol grubu olarak 18 normal endometrium dokusu çalışmaya dahil edilmiştir. Çalışmaya dahil edilen endometrial karsinomlu olguların tamamı endometrioid tip adenokarsinoma olup, olguların yaşları 36 ile 74 (ortalama yaş 57) arasında değişmektedir. Olguların patolojik değerlendirme sonuçlarına göre dağılımları Tablo 4.1'de verilmiştir. Çalışmaya dahil edilen endometrial hiperplazili olguların yaşları ise 33 ile 70 arasında degişmekte olup, ortalama yaş 48 olarak bulunmuştur. Olguların alt gruplara göre dağılımı tablo 4.2'de belirtilmiştir.

Tablo 4.1. Endometrial karsinomlu olguların patolojik değerlendirme sonuçlarına göre dağılımları

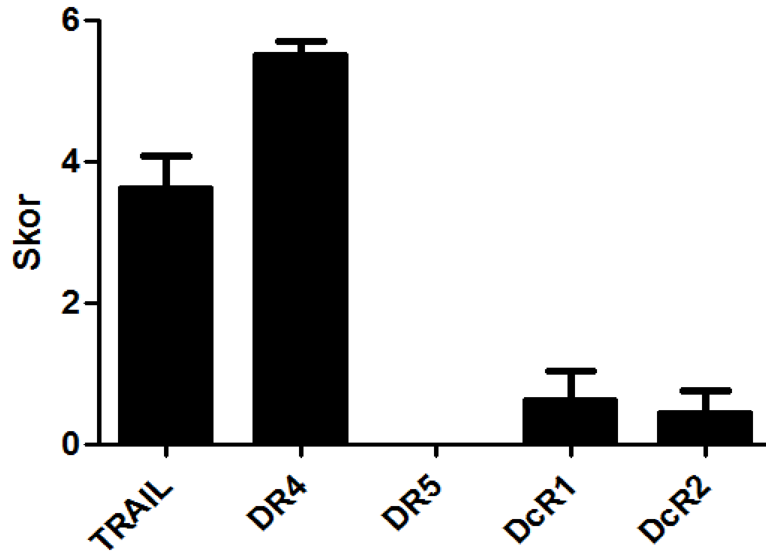
	Hasta Sayısı
Histolojik Tip	
Endometrioid tip adenokarsinoma	100
Tümör Grade (n=100)	
G1	63
G2	26
G3	11
Miyometriyal İnvazyon Derinliği (n=100)	
İnvazyon yok	9
%50'ye eşit veya daha az	45
%50'den fazla	46
Stage (n=100)	
I	68
II	6
III	24
IV	1

Tablo 4.2. Endometrial hiperplazili hastaların alt gruplara göre dağılımı

	Hasta Sayısı
Histolojik Tip	
Endometriyal Hiperplazi	27
Basit	1
Kompleks atipisiz	7
Kompleks atipili	19

4.1. Normal Endometriumda TRAIL Ligand ve Reseptörlerinin Ekspresyon Profili

Çalışmamızın ilk aşamasında kontrol grubunda yer alan 18 normal endometrium dokusundan elde edilen parafin kesitlerde TRAIL ligand ve reseptörleri TRAIL-R1(DR4), TRAIL-R2(DR5), TRAIL-R3(DcR1), TRAIL-R4(DcR2)'e spesifik antikorlar kullanılarak, ekspresyon profillerinin immühistokimyasal olarak değerlendirilmesi yapılmıştır. Bu dokularda yapılan immünohistokimyasal analizlerde immün boyanma skorları her bir doku örneği için boyanma şiddeti ve yaygınlık değeri toplanarak elde edilmiştir. İmmün boyamalara bağlı olarak yapılan skorlama sonucunda normal endometrium grubu için TRAIL ve reseptörleri açısından elde edilen ekspresyon profili Şekil 4.1'de verilmiştir.



Şekil 4.1. Normal Endometriumda TRAIL ligand ve reseptörlerinin ekspresyon profili. Her bir bar 18 normal endometrium dokusuna ait boyanma skorlarının ortalamasını (±SEM) göstermektedir.

Normal endometrium dokularında elde edilen bulgular istatistiksel olarak değerlendirilmiştir. Normalite testi olarak Shapiro-Wilk testi uygulanmış ve grup içi dağılımın Gaussian dağılımına uymadığı (normal dağılım göstermediği) belirlendiği için toplu olarak TRAIL ligand ve reseptörlerinin sentez farklılıklarının istatistiksel olarak anlamlı olup olmadığını belirleyebilmek amacıyla Friedman testi kullanılmıştır. Elde edilen bulgudan hareketle grup içi TRAIL ligand ve reseptör sentez düzeyleri arasında anlamlı bir fark bulunduğu sonucuna varılmıştır. TRAIL ligand ve reseptörlerinin ikili olarak karşılaştırılması için Wilcoxan Signed Rank testi uygulanmıştır. Normal endometrium dokularında yapılan boyamalara bağlı olarak elde edilen verilere dayalı grafiğe bakıldığında en fazla sentez edilen TRAIL

reseptörünün DR4 ölümcül reseptörü olduğu gözlenmiş ve istatistiksel olarak da DR4 ekspresyonunun TRAIL ve diğer reseptörlere göre anlamlı derecede yüksek olduğu belirlenmiştir. En düşük oranda sentez edilenler ise DR5 (ekspresyonu hiç yok), DcR1 ve DcR2 reseptörleri olarak belirlenmiş ve bu üç reseptörün ekspresyon seviyeleri arasında anlamlı bir fark bulunamamıştır.

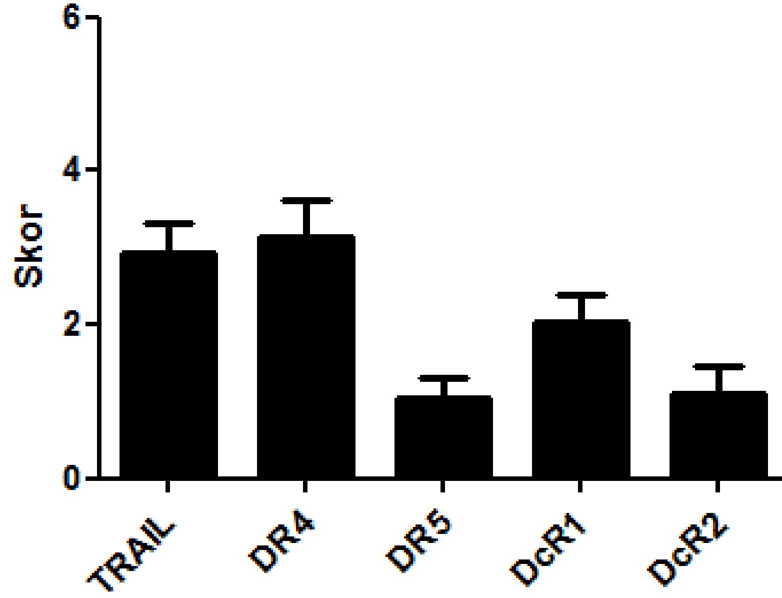
TRAIL ligand ve reseptör ekspresyonları arasındaki olası ilişkileri belirlemek için Spearman Rho Korelasyon testi uygulanmıştır (Tablo 4.3). Spearman's Rho korelasyon testine göre normal endometrium dokularında TRAIL ve reseptörleri arasında pozitif ya da negatif bir korelasyon belirlenememiştir.

Tablo 4.3. Spearman's Rho korelasyon testi ile normal endometriumda TRAIL ligand ve reseptörlerinin sentez profilinin karşılaştırılması

		TRAIL	DR4	DR5	DcR1	DcR2
TRAIL	Correlation Coefficient	1,000	,355		,193	-,064
	Sig. (2-tailed)		,148		,444	,800
	N	18	18	18	18	18
DR4	Correlation Coefficient	,355	1,000		,245	-,100
	Sig. (2-tailed)	,148			,327	,694
	N	18	18	18	18	18
DR5	Correlation Coefficient					
	Sig. (2-tailed)					
	N	18	18	18	18	18
DcR1	Correlation Coefficient	,193	,245		1,000	,434
	Sig. (2-tailed)	,444	,327			,072
	N	18	18	18	18	18
DcR2	Correlation Coefficient	-,064	-,100		,434	1,000
	Sig. (2-tailed)	,800	,694		,072	
	N	18	18	18	18	18

4.2. Endometrial Hiperplazili Hastalarda TRAIL Ligand ve Reseptörlerinin Ekspresyon Profili

Endometrial hiperplazili 27 hastaya ait parafin kesitlerde TRAIL ligand ve reseptörleri TRAIL-R1(DR4), TRAIL-R2(DR5), TRAIL-R3(DcR1), TRAIL-R4(DcR2)'e spesifik antikorlar kullanılarak, ekspresyon profillerinin immünohistokimyasal değerlendirilmesi yapılmıştır. Bu hastalarda immün boyamalara bağlı olarak yapılan skorlama sonucunda bu hasta grubu için TRAIL ve reseptörleri açısından elde edilen ekspresyon profili Şekil 4.2'de verilmiştir.



Şekil 4.2. Endometrial Hiperplazili hastalarda TRAIL ligand ve reseptörlerinin ekspresyon profili. Her bir bar 27 endometrial hiperplazili hastaya ait boyanma skorlarının ortalamasını (±SEM) göstermektedir.

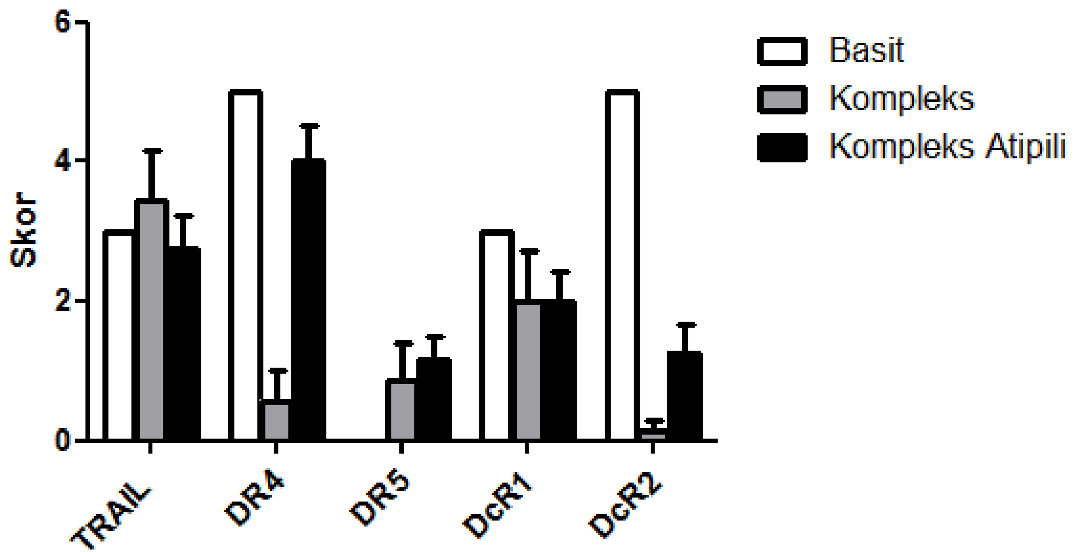
Endometrial hiperplazi dokularında elde edilen bulgular istatistiksel olarak değerlendirilmiştir. Normalite testi olarak Shapiro-Wilk testi uygulanmış ve grup içi dağılımın Gaussian dağılımına uymadığı (normal dağılım göstermediği) belirlendiği için toplu olarak TRAIL ligand ve reseptörlerinin sentez farklılıklarının istatistiksel olarak anlamlı olup olmadığını belirleyebilmek amacıyla Friedman testi kullanılmıştır. Elde edilen verilerden hareketle grup içi TRAIL ligand ve reseptör sentez düzeyleri arasında anlamlı bir farklılık bulunduğu sonucuna varılmıştır. TRAIL ligand ve reseptörlerinin ikili olarak karşılaştırılması için Wilcoxan Signed Rank testi uygulandı. Endometrial hiperplazi dokularında grafiğe bakıldığında en fazla sentez edilen DR4 ölümcül reseptörü iken, istatistiksel olarak DR4 ekspresyonunun TRAIL ($p=0,860$) ve DcR1'e ($p=0,093$) göre anlamlı derecede yüksek olmadığı belirlenmiştir. En düşük oranda sentez edilen reseptörlerin ise DR5 ve DcR2 olduğu saptanmıştır.

TRAIL ligand ve reseptör ekspresyonları arasındaki olası ilişkileri belirlemek için Spearman Rho Korelasyon testi uygulamıştır (Tablo 4.4). Spearman Rho korelasyon testine göre DR4 ile DcR2 reseptörleri arasında ($p<0,01$) ve TRAIL ligandı ile DcR2 reseptörü arasında ($p<0,05$) pozitif korelasyonlar belirlenmiştir.

Tablo 4.4. Spearman's Rho korelasyon testi ile endometrial hiperplazide TRAIL ligand ve reseptör sentez profillerinin karşılaştırılması (*Korelasyon p<0,05 ve **Korelasyon p<0,01)

	TRAIL	DR4	DR5	DcR1	DcR2
TRAIL Correlation Coefficient	1,000	,206	,071	-,102	,395*
Sig. (2-tailed)		,293	,720	,606	,038
N	28	28	28	28	28
DR4 Correlation Coefficient	,206	1,000	,217	-,283	,596**
Sig. (2-tailed)	,293		,267	,144	,001
N	28	28	28	28	28
DR5 Correlation Coefficient	,071	,217	1,000	,131	,209
Sig. (2-tailed)	,720	,267		,507	,285
N	28	28	28	28	28
DcR1 Correlation Coefficient	-,102	-,283	,131	1,000	-,262
Sig. (2-tailed)	,606	,144	,507		,179
N	28	28	28	28	28
DcR2 Correlation Coefficient	,395*	,596**	,209	-,262	1,000
Sig. (2-tailed)	,038	,001	,285	,179	
N	28	28	28	28	28

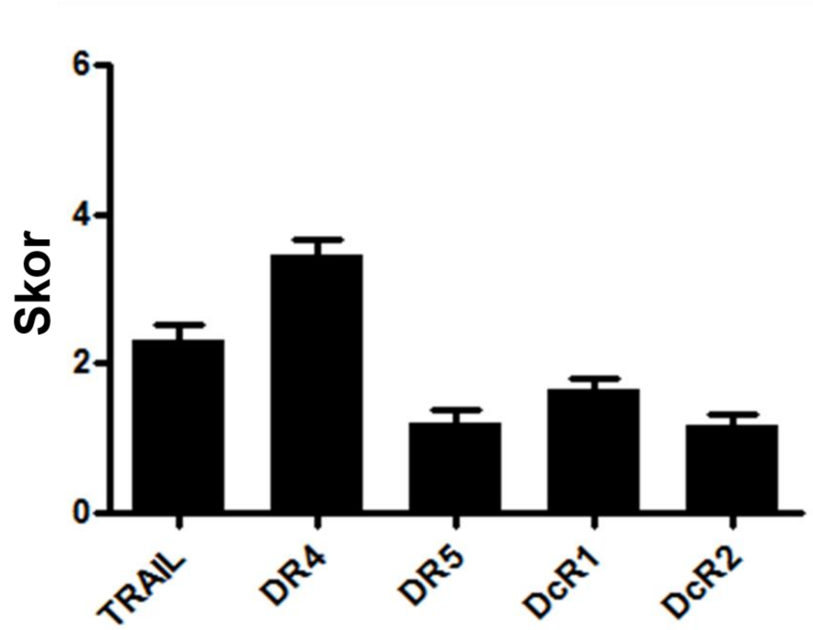
Endometrial hiperplazi dokuları basit, kompleks ve kompleks atipili olmak üzere alt gruplara ayrılarak da TRAIL ligand ve reseptörlerinin ekspresyon profilleri açısından değerlendirilmiştir (Şekil 4.3).



Şekil 4.3. Endometrial Hiperplazi alt gruplarında TRAIL ligand ve reseptörlerinin ekpresyon profili. (Basit hiperplazi n:1, Kompleks Hiperplazi n:7, Kompleks Atipik Hiperplazi n:19)

4.3. Endometrioid Tip Adenokarsinomlu Hastalarda TRAIL Ligand ve Reseptörlerinin Ekspresyon Profili

Endometrioid tip adenokarsinomlu 100 hastaya ait parafin kesitlerde TRAIL ligand ve reseptörleri TRAIL-R1(DR4), TRAIL-R2(DR5), TRAIL-R3(DcR1), TRAIL-R4(DcR2)'e spesifik antikorlar kullanılarak, ekspresyon profillerinin immühistokimyasal olarak değerlendirilmesi yapılmıştır. Bu hastalarda immün boyamalara bağlı olarak yapılan skorlama sonucunda bu hasta grubu için TRAIL ve reseptörleri açısından elde edilen ekspresyon profili Şekil 4.4'de verilmiştir.



Şekil 4.4. Endometrioid tip adenokarsinomalı hastalarda TRAIL ligand ve reseptörlerinin ekpresyon profili. Her bir bar 100 endometrioid tip adenokarsinomalı hastaya ait boyanma skorlarının ortalamasını (±SEM) göstermektedir.

Endometrioid tip adenokarsinoma dokularında elde edilen bulgulardan yola çıkılarak yapılan istatistiksel değerlendirmelerde normalite testi olarak Kolmogorov-Smirnov testi uygulanmış ve grup içi dağılımın Gaussian dağılımına uymadığı belirlenmiştir. Bu nedenle TRAIL ligand ve reseptörlerinin sentez farklılıklarının istatistiksel olarak anlamlı olup olmadığının analizinde Friedman testi kullanılmıştır. Elde edilen veriler, grup içi TRAIL ligand ve reseptör sentez düzeyleri arasında anlamlı bir fark bulunduğunu göstermiştir. TRAIL ligand ve reseptörlerinin ikili olarak karşılaştırılması için Wilcoxan Signed Rank testi uygulanmıştır. Endometrioid tip adenokarsinomda en fazla sentezlenen TRAIL reseptörü DR4 iken, en düşük oranda sentez edilen reseptörlerin ise DR5 ve DcR2 olduğu saptanmıştır. DR5 ve DcR2 reseptör ekspresyonları arasında istatistiksel olarak anlamlı bir farklılık belirlenmemiştir (p=0,958). Reseptörler kendi içinde karşılaştırıldıklarında DR4

ölüm reseptörü DR5’e göre (p=0,000), DcR1 yalancı reseptörü ise DcR2’ye göre istatistiksel olarak daha yüksek düzeyde belirlenmiştir (p=0,019).

TRAIL ligand ve reseptör ekspresyonları arasındaki olası ilişkileri belirlemek için Spearman Rho Korelasyon testi uygulanmıştır (Tablo 4.5). Spearman Rho korelasyon testine göre DR5 ve DcR1 reseptörleri arasında pozitif korelasyon belirlenirken (p<0,01), DR4-DR5 arasında negatif ve DcR1-DcR2 arasında pozitif korelasyonlar (p<0,05) belirlenmiştir.

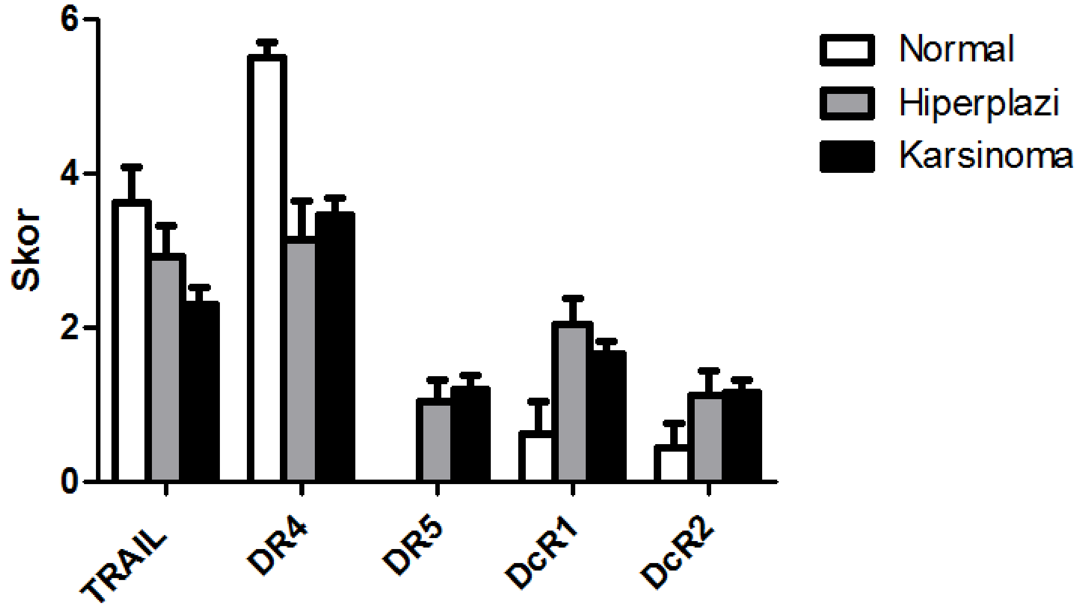
Tablo 4.5. Spearman’s Rho korelasyon testi ile endometrioid tip adenokarsinomada TRAIL ligand ve reseptörlerinin sentez profilinin karşılaştırılması (*Korelasyon p<0,05 ve **Korelasyon p<0,01)

	TRAIL	DR4	DR5	DcR1	DcR2
TRAIL Correlation Coefficient	1,000	,144	,033	,021	-,123
Sİg. (2-tailed)		,154	,743	,835	,225
N	100	100	100	100	100
DR4 Correlation Coefficient	,144	1,000	-,246*	-,030	,133
Sİg. (2-tailed)	,154		,014	,770	,188
N	100	100	100	100	100
DR5 Correlation Coefficient	,033	-,246*	1,000	,274**	,084
Sİg. (2-tailed)	,743	,014		,006	,405
N	100	100	100	100	100
DcR1 Correlation Coefficient	,021	-,030	,274**	1,000	,236*
Sİg. (2-tailed)	,835	,770	,006		,018
N	100	100	100	100	100
DcR2 Correlation Coefficient	-,123	,133	,084	,236*	1,000
Sİg. (2-tailed)	,225	,188	,405	,018	
N	100	100	100	100	100

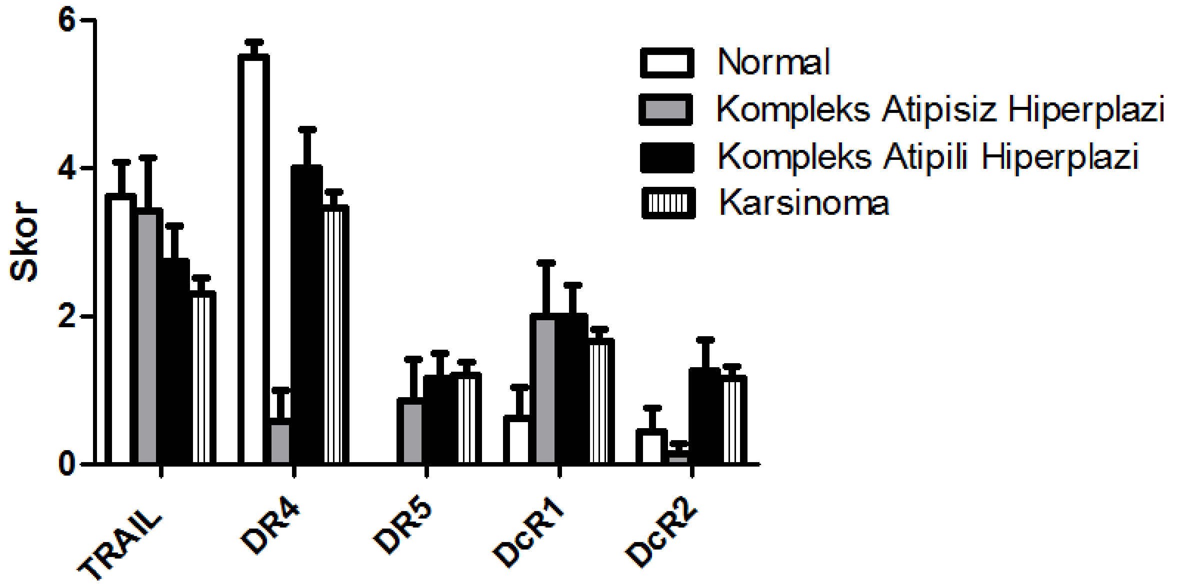
4.4. Normal, Hiperplazi ve Endometrial Karsinoma Örneklerinde TRAIL ve TRAIL Reseptörlerinin Karşılaştırmalı Ekspresyon Profilleri

Endometrial karsinoma, hiperplazi (kompleks atipisiz ve kompleks atipili hiperplazi) ve normal endometrium dokularında immunohistokimyasal olarak değerlendirilmesi yapılan TRAIL ligand ve reseptörlerinin ekspresyon profilleri Şekil 4.5 ve Şekil 4.6’da, her bir antikor için yapılan immün boyamalara ait örnek görüntüler ise Şekil 4.7’de karşılaştırmalı olarak verilmiştir.

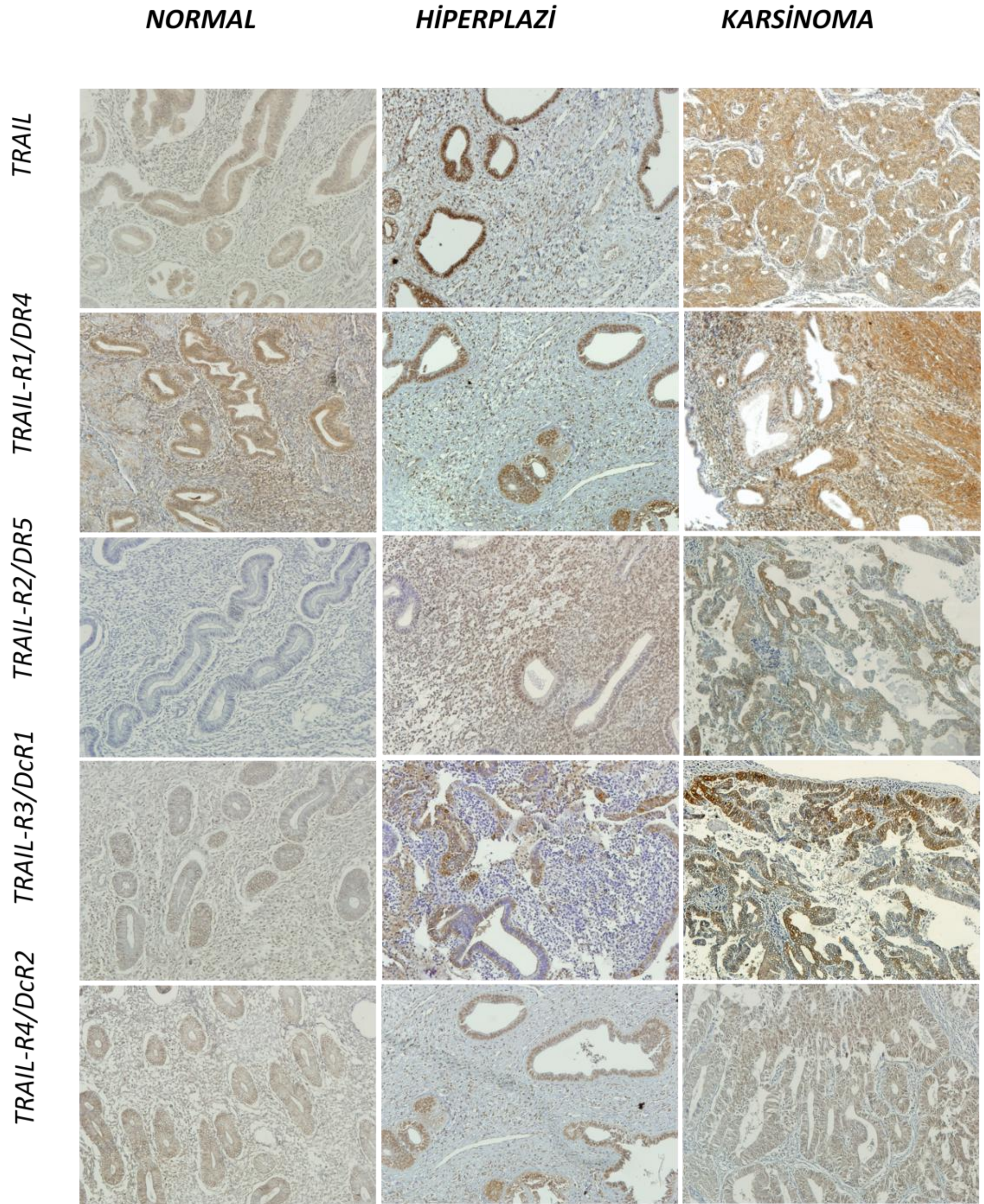
Gruplar arasında TRAIL ligand ve reseptör ekspresyonları açısından anlamlı bir fark olup olmadığını göstermek üzere Kruskal-Wallis testi uygulanmış ve tüm gruplarda TRAIL ligand ve reseptör ekspresyonları arasında istatistiksel olarak anlamlı bir fark belirlenmiştir. Normal, Kompleks atipisiz, Kompleks atipili hiperplazi ve Karsinoma örnekleri karşılaştırıldığında ise yine TRAIL reseptörlerinin ekspresyon seviyelerinde anlamlı bir fark gözlenmiş, fakat TRAIL ligand ekspresyon düzeylerinde böyle bir farklılığın söz konusu olmadığı belirlenmiştir.



Şekil 4.5. Normal, Hiperplazi ve Endometrial karsinomada TRAIL ligand ve reseptör ekspresyon profillerinin karşılaştırmalı analizi.



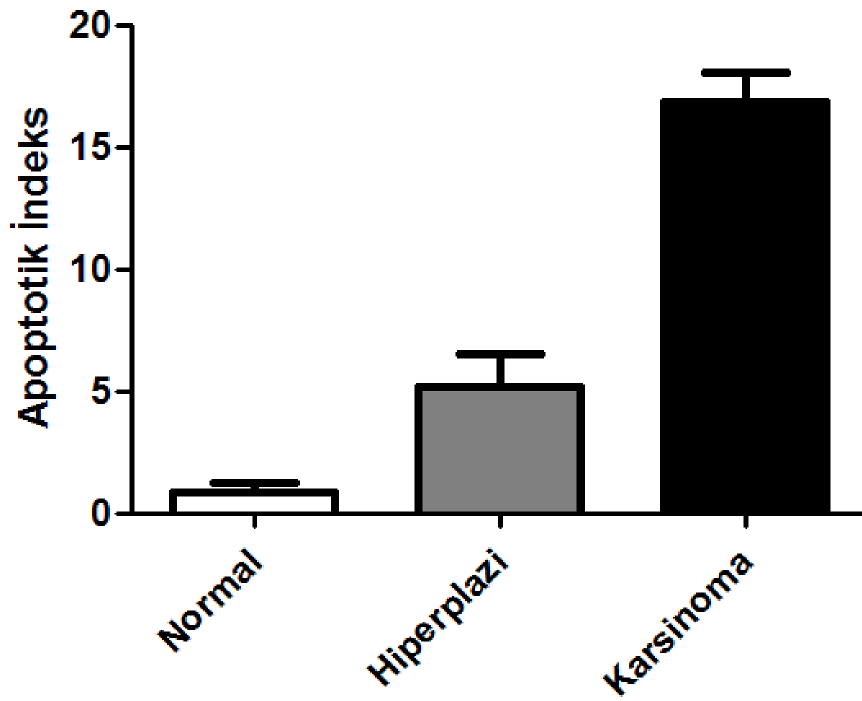
Şekil 4.6. Normal, Kompleks atipili, Kompleks atipisiz hiperplazi ve Endometrial karsinoma örneklerinde TRAIL ligand ve reseptör ekspresyon profillerinin karşılaştırmalı analizi.



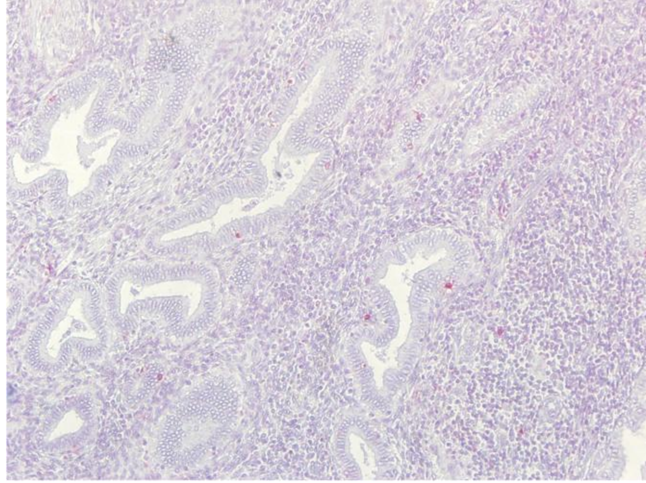
Şekil 4.7. Normal endometrium (Sol Panel), Endometrial Hiperplazi (Orta Panel) ve Endometrioid tip adenokarsinoma (Sağ Panel) dokularında immünohistokimyasal boyama örnekleri. Kahverengi presipitat pozitif boyanmayı göstermektedir. Her bir panelin sol tarafında boyamada kullanılan antikorlar belirtilmiştir. Mikroskop büyütmesi 200X.

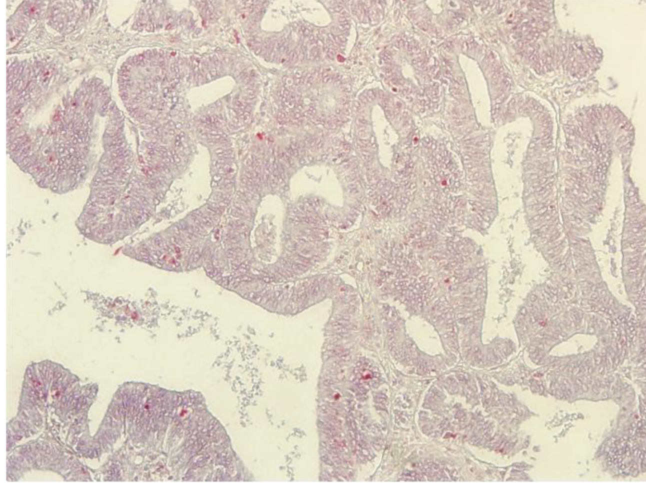
4.5. Normal, Hiperplazi ve Endometrial Karsinomda Apoptozisin Değerlendirilmesi

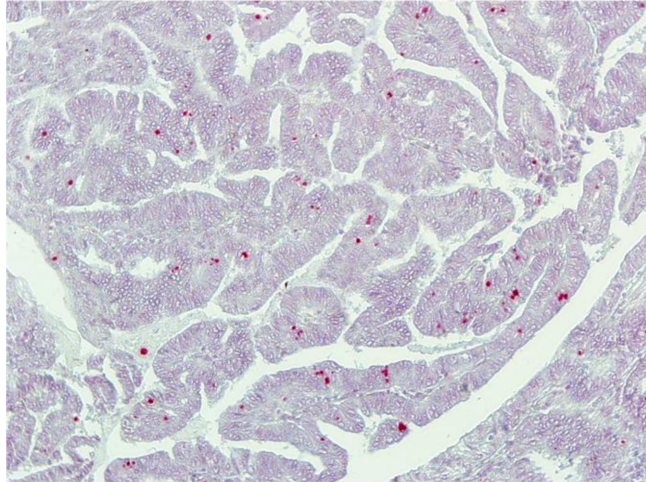
Normal endometrium, Endometrial hiperplazi ve Endometrial karsinomda apoptozisin belirlenebilmesi için üç gruba ait toplam 145 olguya ait parafin kesitte materyal ve metod bölümünde açıklandığı gibi TUNEL boyamaları yapıldı. TUNEL boyamalarını takiben her bir preparatta rastgele odaklanılan 10 ayrı alanda metodun son basamağında kullanılan Fast-Red boyası ile kırmızıya boyanan apoptotik hücreler değerlendirilmiş ve bu sayının toplam hücre sayısına bölünmesiyle ortaya çıkan oranların ortalaması alınmıştır. Bu oran 100 ile çarpılarak % Aİ (Apoptotik İndeks) değeri elde edilmiştir. Normal endometrium, Endometrial hiperplazi ve Endometrial karsinoma dokularında elde edilen apoptotik indeks oranları Şekil 4.8'de gösterilmiştir. TUNEL boyamalara ait örnek görüntüler Şekil 4.9'da verilmiştir.



Şekil 4.8. Hücre ölümlerinin kantitatif analizi. Apoptotik hücrelerin varlığı TUNEL yöntemi ile değerlendirilmiştir. Her bir bar gruplara ait boyanma skorlarının ortalamasını (± SEM) göstermektedir. Normal endometrium n:18, Endometrial Hiperplazi n:27, Endometrial Karsinoma n:100







Şekil 4.9. Endometrium dokularında TUNEL boyama örnekleri. Kırmızı renk boyanmalar apoptotik hücreleri göstermektedir. Mikroskop büyütmesi 200X.

Normal endometrium, Endometrial hiperplazi ve Endometrial karsinomda TRAIL ligand ve reseptör ekspresyon profilleriyle hücre ölümleri arasında korelasyon olup olmadığı yine Spearman Rho korelasyon testiyle belirlendi. Tablo 4.6'da gösterildiği gibi TUNEL pozitif hücre sayısıyla TRAIL ligand ve reseptörleri arasında herhangi bir korelasyon saptanamamıştır.

Tablo 4.6. Spearman's Rho korelasyon testi ile normal, hiperplazi ve karsinoma dokularında TUNEL pozitif hücre sayısı ile TRAIL ligand ve reseptörlerinin ekspresyon profillerinin karşılaştırılması

			Normal TUNEL	Hiperplazi TUNEL	Karsinoma TUNEL
Spearman's rho	TUNEL	Correlation Coefficient	1,000	,1000	1000
		Sİg. (2-tailed)			
		N	17	26	97
	TRAIL	Correlation Coefficient	-,343	,025	-,120
		Sİg. (2-tailed)	,178	,904	,240
		N	17	26	97
	DR4	Correlation Coefficient	,264	-,375	-,006
		Sİg. (2-tailed)	,306	,059	,950
		N	17	26	97
	DR5	Correlation Coefficient	-	,254	,109
		Sİg. (2-tailed)	-	,210	,288
		N	17	26	97
	DcR1	Correlation Coefficient	-,293	,350	-,029
		Sİg. (2-tailed)	,253	,079	,780
		N	17	26	97
	DcR2	Correlation Coefficient	-,005	-,211	,001
		Sİg. (2-tailed)	,985	,300	,990
		N	17	26	97

4.6. TRAIL Ligand ve Reseptör Ekspresyon Profilinin Klinik ve Patolojik Parametreler ile Değerlendirilmesi

TRAIL ligand ve reseptör ekspresyon profili ile hastalığın klinik evresi, histolojik derece ve myometriyal invazyon arasındaki ilişki yine Spearman Rho korelasyon testiyle analiz edilmiştir. Analiz sonuçlarına göre TRAIL ligand ve reseptör ekspresyon profiliyle endometrial karsinomlu hastaların klinik evrelemesi, histolojik derece ve myometriyal invazyon derinliği arasında istatiksel olarak anlamlı bir ilişki bulunamamıştır (Tablo 4.7)

Tablo 4.7. Endometriod tip adenokarsinomada Spearman's Rho korelasyon testi ile TRAIL ligand ve reseptör profilinin klinik ve patolojik parametreler ile karşılaştırılması

			Evre (Stage)	Derece (Grade)	Myometrial İnvazyon
Spearman's rho	TRAIL	Correlation Coefficient	-,084	-,097	-,125
		Sig. (2-tailed)	,408	,338	,215
		N	99	100	100
	DR4	Correlation Coefficient	,059	,114	-0,42
		Sig. (2-tailed)	,559	,258	,678
		N	99	100	100
	DR5	Correlation Coefficient	,073	,172	-,026
		Sig. (2-tailed)	,473	,087	,796
		N	99	100	100
	DcR1	Correlation Coefficient	-,006	,035	-,048
		Sig. (2-tailed)	,951	,728	,636
		N	99	100	100
	DcR2	Correlation Coefficient	-,010	,075	117
		Sig. (2-tailed)	,918	,456	,245
		N	99	100	100

TARTIŞMA

Endometrium kanseri kadın genital sistem kanserleri arasında en sık rastlanan kanser tipidir. Diğer kanserlerde olduğu gibi jinekolojik kanserlerde de erken tanı, etkili ve potansiyel tedavi imkânları sağlamaktadır. Endometrium kanserli olgular, genellikle evre I'de semptomatik olarak başvuran ileri yaşta kadınlardır. Etkin bir tarama testi olmamasına karşın, olguların % 75-88'i semptom vermesi nedeniyle erken evrede teşhis edilebilmektedir. Bu hastaların % 80-85'i 5 yıllık sağ kalım oranı ile iyi prognoza sahiptir. Ancak, tedavi edilen hastaların % 15-20'sinde hastalık tekrarlayabilmekte ve bu hastalar hayatını kaybetmektedir [107]. İleri evre olgularda ise tedavi başarı oranı daha da düşmektedir. Bu durumda hastalığın prognozunun tespiti ön plana çıkmaktadır. Özellikle, ileri evre olgularda prognozu önceden belirlemede prognostik faktörlere olan gereksinim, son yıllarda ilgi odağı haline gelmiştir. Dolayısıyla endometrium kanseri de, jinekolojik kanserler içerisinde prognostik parametreleri en çok araştırılan kanserlerden birisi olmuştur.

Endometrial karsinomalarda tanımlanmış en önemli klinikopatolojik prognostik faktörler yaş, histolojik tip, evre, grade, myometrial invazyon derinliği, peritoneal sitoloji, adneksiyal tutulum, servikal tutulum, vasküler invazyon, seks steroid reseptör durumu, intraperitoneal metastazlar, lenf nodu metastazları şeklinde özetlenebilir [34]. Bilinen prognostik faktörlerin yanı sıra hastalığın klinik progresyonunda önemli olduğu düşünülen çok sayıda moleküler biomarker üzerinde de çalışılmıştır. Bu moleküler faktörler, neovaskülarizasyon/vasküler proliferasyon/vasküler endotelyal büyüme faktörleri, p53, anti-apoptotik faktörler, proliferasyon faktörleri, steroid reseptörleri, p16, plazminojen aktivatör inhibitörü ve tümör ilişkili makrofajlardır [108].

Gerek endometrium kanseri, gerekse diğer kanserlerde mevcut tedavi yöntemlerinin geliştirilebilmesi ve yeni tedavi metodlarının bulunması için hastalığın moleküler patogenezinin açıklanması gerekmektedir. Bu hedefe yönelik olarak son yıllarda en çok irdelenen parametrelerden biri TRAIL ligandı ve reseptörleridir. Kanserde TRAIL ligand ve reseptör profillerinin belirlenmesi, TRAIL aracılı apoptoz indüksüyonu ve kanser tedavisinde kullanılan ilaçlara verilecek cevabın önceden belirlenmesinde önem taşımaktadır. Diğer taraftan, TRAIL ligand ve reseptör ekspresyon profillerinin farklı kanser tiplerinde değişkenlik göstermesi nedeniyle, bu profillerin hastalığın prognozunu belirlemede önemli bir belirteç teşkil edebileceği düşünülmektedir.

Birçok farklı hücre hattında TRAIL indüklü apoptozis *in vitro* olarak çalışılmış olmasına rağmen *in vivo* ortamda TRAIL sinyal yolağı ve TRAIL reseptör ekspresyonlarının rolü yeterince aydınlatılamamıştır. Son yıllarda yapılan çalışmalara bakıldığında ise TRAIL ligand ve reseptör ekspresyon profillerinin

kanser prognozuyla ilişkisini araştıran çalışmaların sayısının oldukça hızlı bir artış gösterdiği göze çarpmaktadır. Yakın dönemde yapılan çalışmalarda TRAIL ligand ve reseptörleri hepatosellüler karsinoma, kolon kanseri, prostat kanseri, pankreas kanseri, küçük hücreli olmayan akciğer kanseri ve meme kanseri gibi farklı kanser tiplerine ait doku örneklerinde değerlendirilmiştir. Bu konuda örnek çalışmaların başında grubumuz tarafından, 90 meme kanserli hastaya ait dokuda TRAIL ligand ve reseptörlerinin ekpresyonunu belirlemeye yönelik olarak gerçekleştirilmiş çalışma gelmektedir. Bu çalışmada, meme kanser dokularında ölüm reseptörü DR4 ekspresyonu en yüksek düzeyde belirlenirken, DcR1 dekoy reseptör ekspresyonu en düşük olarak belirlenmiştir. Bu dokularda DcR2 ekpresyonu belirgin bir şekilde mevcut olduğu halde, DR5 ve TRAIL ligand ekspresyon düzeyinden istatistiksel anlamda daha yüksek olarak bulunmamıştır. Bu hastalarda aynı zamanda menopoz durumu, progesteron ve östrojen reseptörleri ve CerbB2 ekspresyonu ile TRAIL ve reseptörlerinin ilişkisi araştırılmıştır. Sonuç olarak, kötü prognoz ile ilişkili olduğu bilinen progesteron reseptörü açısından negatif olan hastalarda DR5 ekspresyonu yüksek olarak belirlenirken, yine kötü prognoz ile ilişkili CerbB2 pozitif hastaların CerbB2 negatif hastalara nazaran daha yüksek TRAIL ve DR5 ekspresyon seviyesine sahip olduğu saptanmıştır. Benzer bir şekilde, premenapozal dönem meme kanser hastalarında da yüksek TRAIL ekspresyon seviyesinin söz konusu olduğu belirlenmiştir. Bu hastalarda, tümör grade ile pozitif korelasyon gösteren tek belirtecin yüksek düzeyde DR4 ekspresyonu olduğu tespit edilmiştir. Bu nedenle DR4'ün meme karsinogenezisinde önemli bir rol oynadığı ve aynı zamanda prognostik bir belirteç olarak kullanılabileceği düşünülmektedir [105]. Meme kanserinde TRAIL reseptör ekspresyonunun prognostik önemini açıklamaya yönelik yapılmış geniş çaplı bir başka çalışmada ise 311 hastaya ait meme kanser dokusunda immunohistokimyasal analizler yapılmıştır. Bu çalışmada iyi diferansiye tümörlerde yüksek düzeyde DR4 ekspresyonu belirlenmiş ve DR4 ekspresyonu hormon reseptör durumu, Bcl-2, negatif nodal durum gibi iyi prognostik belirteçler ile pozitif ilişkili, Her2/neu ekspresyonu ve proliferasyon markırı Ki67 ile negatif ilişkili olarak belirlenmiştir. Bu durumun aksine, DR5 ve DcR2 ekspresyonu yüksek tümör grade, yüksek Ki67 ve Her2/neu ekspresyonu, pozitif nodal durum ve düşük Bcl-2 ekspresyonu ile ilişkili bulunmuştur. Bunlara ek olarak bu iki reseptörün ekspresyonunun meme kanserli hastaların sağ kalımı ile negatif korelasyon gösterdiği belirtilmiştir [109].

Prostat kanserinde TRAIL ve TRAIL reseptörlerinin *in vivo* ekspresyon profillerini belirlemek üzere; 44 benign prostatik hiperplazi (BPH), 28 Organa sınırlı prostat karsinoma (OCPCa) ve 26 ileri evre prostat karsinomalı (APCa) hastaya ait parafine gömülü dokudan alınan kesitlerde immünohistokimyasal analizler gerçekleştirilmiştir. Bu çalışmada tüm hastalarda TRAIL ligand ve reseptörlerinin değişen derecelerde ekspresyonlarının varlığı gösterilmekle beraber tüm hasta gruplarında DcR2 ekspresyonu yüksek olarak belirlenmiştir. Yapılan analizler sonucunda malign prostat tümörlü hastalarda BPH'lı hastalara göre TRAIL ligand ve reseptör ekspresyonları yüksek olarak bulunmuştur. Bu sonuca dayalı olarak araştırmacılar, TRAIL ligand ve reseptör ekspresyon profillerinin prostat kanserinde benign formu malign formdan ayırt etmede kullanılabilecek uygun prognostik belirteçler olarak kullanılabileceğini öne sürmektedir [103]. Grubumuz tarafından yapılan bir prostat kanser çalışmasında ise BPH, OCPCa ve APCa hasta gruplarından

oluşan 98 hastada TRAIL ligand ve reseptörlerinin önemli prognostik belirteçler olan Gleason skor ve serum prostat spesifik antijen (PSA) düzeyi arasındaki ilişki araştırılmıştır. Bu hastalarda serum PSA düzeyleri ile TRAIL ve TRAIL reseptör ekspresyonları arasında pozitif bir korelasyonun varlığının yanı sıra, Gleason skor ile DcR2 reseptörünün yüksek ve TRAIL'ın düşük düzeyde ekspresyonları ilişkili olarak bulunmuştur. Prostat karsinomalı hastalarda kötü prognozun göstergesi olan PSA rekürensi DcR2 reseptör ekspresyonu ile ilişkili olarak belirlenmiş, yüksek DcR2 reseptör ekspresyonu ve düşük TRAIL ölüm ligand ekspresyonu ise azalan sağkalım ile ilişkili bulunmuştur [104].

TRAIL ve reseptörlerinin prognostik önemi ile ilişkili en çok üzerinde çalışılan kanser tiplerinden biri de kolon kanseridir. Strater ve arkadaşlarının normal kolon mukozası (n=10), kolon adenoma (n=20) ve stage II/III kolon karsinoma dokularında (n=129) yapmış olduğu çalışmada normal kolon yüzey epitelyum hücrelerinde TRAIL, DR4, DR5 ve DcR2 ekspresyonu belirlenirken, DcR1 ekspresyonu belirlenememiştir. Bu ekspresyon paterni büyük oranda düşük grade adenomalardaki durumu da yansıtmaktadır. Ancak farklı olarak DcR1 ekspresyonu adenoma ve karsinomalarda eksprese edilmektedir. DcR1 ve DcR2 dekoy reseptör ekspresyonlarının normal mukozadan adenoma ve karsinomaya doğru gelişiminde artışı gösterilmiş fakat bu reseptörlerin ekspresyonu kolon kanserinde kötü prognoz ile ilişkilendirilememiştir [110]. Koornstra ve arkadaşlarının yapmış olduğu çalışmada ise Strater ve arkadaşlarının yapmış olduğu çalışmadan farklı olarak normal mukoza, adenoma ve karsinomaların tümünde DcR1 ekspresyonu belirlenmiştir. Bu çalışmada DR4 ve DR5 ekspresyonu tümör dokularında normal dokulara göre daha yüksek olarak belirlenmekle beraber TRAIL ve TRAIL reseptör ekspresyonları ile histopatolojik özellikler arasında bir korelasyon belirlenmemiştir [98].

Grubumuz tarafından yapılan bir diğer çalışmada ise TRAIL ve TRAIL reseptörlerinin pankreatik karsinoma gelişimindeki rolü araştırılmıştır. Bu çalışma, 31'i sağlıklı ve 34'ü pankreatik duktal adenokarsinomalı hastalardan alınan pankreas dokularında gerçekleştirilmiş ve ekspresyon profillerinin prognostik faktörler ile olan ilişkisi araştırılmıştır. Pankreatik karsinomalı hastalara ait dokularda normal dokulara göre DcR2 ve DR4 reseptör ekpresyonlarında artış ve beraberinde bu hastaların duktal hücrelerinde apoptozis yüzdesinde önemli bir artış gözlenmiştir. Ayrıca, hastaların asinar hücrelerinde yüksek DcR2 ve düşük DR4 ekspresyonu, Langerhans adacıklarında ise artmış DcR2 ekspresyonu gözlenmiştir. Bu sonuçlar, TRAIL ve TRAIL reseptör ekpresyonlarının pankreatik karsinoma gelişiminde önemli bir rol oynuyor olabileceğini göstermektedir [111].

Jinekolojik kanserlerde TRAIL ve reseptörlerinin prognostik önemini belirlemeye yönelik yapılmış çalışmalardan biri serviks kanserlerinde TRAIL, DR4 ve DR5 reseptörlerinin immünohistokimya ile analiz edildiği 645 serviks kanserli hastaya ait dokunun değerlendirildiği çalışmadır. DR4, DR5 ve TRAIL ekspresyonu serviks tümörlerinde yüksek oranlarda belirlenmiştir. Fakat DR4, DR5 ve TRAIL ekspresyonunun hastalık ilişkili sağ kalım için prognostik olmadığı sonucuna

varılmıştır [112]. Ovaryum kanserinde ise belirlenen yüksek TRAIL ekspresyonu daha iyi sağkalım ile ilişkili bulunmuştur [113].

Kanser tedavisinde, TRAIL kullanımında tedaviye cevabın sağlanmasında da TRAIL ligand ve reseptör ekspresyon profillerinin belirlenmesinin önemine yönelik literatürde sayıları gün geçtikçe artan çalışmalar mevcuttur. Bu çalışmalardan birine örnek olarak, stage III küçük hücreli akciğer kanserli hastalarda rekombinant TRAIL'ın terapötik etkisini araştırmaya yönelik yapılan çalışmada incelen tümör dokularında DR4, DR5 ve TRAIL ekspresyon düzeyleri sırasıyla % 99, % 82 ve % 91 olarak belirlenmiştir. Araştırmacılar bu sonuçtan yola çıkarak kemoterapi ve radyoterapiye dirençli bu hastalarda iki ölüm reseptöründen birinin bulunmasının rekombinant TRAIL'in kanser tedavisi için potansiyel bir değere sahip olduğu sonucunu çıkarmıştır [114]. Nitekim biz de akciğer kanserinde yaptığımız çalışmalardan birinde, A549 hücre hattında yüksek DcR2 ekspresyonu saptamış, aynı zamanda DcR2 ekspresyonu ile bu hücrelerde karşılaşılan TRAIL dirençliliği arasında bir ilişki bulunduğunu belirlemiştik. Bu verilerden yola çıkarak DcR2'ye karşı siRNA uyguladığımızda ise bu hücrelerin TRAIL duyarlı hale getirilebildiğini gösterdik. Bu çalışmada ayrıca küçük hücreli olmayan akciğer kanserli 10 hastaya ait dokuda da TRAIL ve reseptörlerinin ekspresyon düzeyleri değerlendirilmiş ve DcR2 ekpresyonu yüksek olarak bulunmuştur [86]. Benzer şekilde, DcR2 ekspresyonunun MCF7 meme kanser hücre hattında da yüksek olduğunu bir başka çalışmamızda ortaya çıkarmıştık. [84]. Endometrial kanser hücre hatlarında ve endometrial orjinli normal epitelyal hücrelerde TRAIL'ın etkisini araştırmak üzere yapılmış bir çalışmada ise Ishikawa ve HEC1a endometrial karsinoma hücre hatları ile endometrial sarkomada TRAIL aracılı apoptozise duyarlılık ile karşılaşılırken normal endometrium hücrelerinde dirençlilik ile karşılaşılmıştır. Bu hücrelerde yapılan RT-PCR analizlerinde, Ishikawa hücrelerinde DcR1 ekspresyonu ve normal endometrium epitelyumunda DR5 yokluğu dışında hepsinde ölüm reseptörleri ve dekoy reseptörlerin varlığı ile karşılaşılmıştır. Sonuç olarak, TRAIL aracılı apoptozis ve dekoy reseptör ekspresyon varlığı ya da yokluğu arasında bir korelasyon bulunamamıştır [115].

Literatürde endometrium kanseri ve endometrial hiperplazilerde TRAIL ligand ve reseptör ekspresyon profillerini belirlemeye yönelik yapılmış çalışma bulunmamaktadır. Bu alanda bilgi verici Tarragona ve arkadaşları tarafından yapılmış tek bir çalışma mevcuttur ki bu çalışmada da sadece DcR1 ekspresyon durumu değerlendirilmiştir. Bu çalışmada, 80 normal endometrium, 62 endometrial karsinoma dokusunda immunohistokimyasal ve 19 normal endometrium ve 28 endometrial karsinoma dokusunda real-time RT-PCR ile değerlendirme yapılmıştır. İmmunohistokimya ile yapılan değerlendirmelerde normal endometrium dokularında DcR1 ekspresyonu % 79,6 olarak belirlenirken histolojik tip, grade ve evre ile istatistiksel bir ilişki kurulmaksızın endometrial karsinoma vakalarının % 98,1'inde belirlenmiştir. Real time RT-PCR sonuçlarına göre ise DcR1 mRNA'sı tüm normal endometrium dokularında bazal düzeyde benzer olarak belirlenirken, endometrial karsinoma dokularında bazal düzeyin yaklaşık 5 katı artış saptanmıştır (28 vakanın 13'ünde (% 46,4)). Yüksek DcR1 ekspresyon düzeyleri evre IA'da 12 vakanın 4'ünde (% 33); IB'de 4 vakanın 2'sinde (% 50); IC'de 6 vakanın 4'ünde (% 66); ve

IIA ve IIB'de 6 vakanın 3'ünde (%50) olmak üzere farklı evrelerde belirlenmiştir. Bu sonuca dayalı olarak endometrial karsinomada DcR1 ekspresyonu varlığının TRAIL-aracılı apoptozise dirençliliğe katkıda bulunduğu düşünülmektedir [116].

Yapmış olduğumuz çalışmada 100 endometrial karsinomlu, 27 endometrial hiperplazili olguya ait doku ve kontrol grubu olarak da 18 normal endometrium dokusu, TRAIL ligand ve reseptörlerinin ekspresyon profillerini belirlemek üzere immünohistokimyasal olarak değerlendirilmiştir. Endometrium kanser olgularının yaklaşık % 80'ini endometrioid tip adenokarsinomlar oluşturmaktadır. İncelemiş olduğumuz 100 endometrial karsinomlu olguya ait dokuların tümü, endometrial kanserlerin genel durumunu yansıtması açısından histolojik olarak endometrioid tip adenokarsinomalı hastalardan oluşturulmuştur. Çalışmaya alınan 100 hastanın yaş ortalaması 57'dir.

Doku örneklerinde immünohistokimya yöntemi ile TRAIL ligand ve reseptörleri olan TRAIL-R1(DR4), TRAIL-R2(DR5), TRAIL-R3(DcR1), TRAIL-R4(DcR2) ekspresyonları spesifik antikorlar kullanılarak değerlendirilmiştir. Endometrioid tip adenokarsinoma örneklerinde hem TRAIL hem de TRAIL reseptörlerinin değişen düzeylerde ekspresyonlarına rastlanmıştır. Bu hastalarda DR4 ölümcül reseptörü ve TRAIL ölüm ligandı skor ortalaması diğer belirteçlere göre daha yüksek olarak belirlenmiştir. En fazla sentez edilen DR4 ölümcül reseptörü olurken, en düşük oranda sentez edilenler ise DR5 ve DcR2 reseptörleri olmuştur. DR5 ve DcR2 reseptör ekpresyonları arasında istatiksel olarak anlamlı bir farklılık belirlenmemiştir. Reseptörler kendi içinde karşılaştırıldıklarında DR4 ölüm reseptörü DR5'e göre, DcR1 yalancı reseptörü ise DcR2'ye göre istatistiksel olarak daha yüksek düzeyde belirlenmiştir. TRAIL ligand ve reseptör ekspresyonları arasındaki olası ilişkileri belirlemek üzere Spearman's Rho korelasyon testi uygulanmıştır. Spearman's Rho korelasyon testine göre DR5 ve DcR1 reseptörleri arasında pozitif korelasyon belirlenirken ($p<0,01$), DR4-DR5 arasında negatif ve DcR1-DcR2 arasında pozitif korelasyonlar ($p<0,05$) belirlenmiştir. Bu sonuçlar DR5 ölüm reseptörü artış gösterirken beraberinde DcR1, aynı şekilde DcR1 reseptörü artış gösterirken beraberinde DcR2 reseptörünün artış gösterdiğini, diğer taraftan DR4 ölüm reseptörü artış gösterirken DR5 ölüm reseptörünün azaldığını işaret etmektedir.

Kontrol grubu olarak çalışılan normal endometrium dokuları ile endometrioid tip adenokarsinoma dokularında, TRAIL ligand ve reseptör ekspresyonları karşılaştırılmış ve iki grup arasındaki istatiksel fark Mann-Whitney U test ile değerlendirilmiştir. Sonuç olarak karsinoma dokularında normal endometriuma göre TRAIL ligandı ($p=0,019$) ve DR4 ölüm reseptöründe ($p=0,000$) azalma, DR5 ölüm reseptörü ($p=0,003$), DcR1 ($p=0,000$) ve DcR2 ($p=0,021$) dekoy reseptörlerinde artış belirlenmiştir. Bilindiği üzere TRAIL ölüm ligandı ölüm reseptörleri ile ilişkiye girerek hücreleri apoptozise götürebilmektedir. Bu nedenle normal hücreden kanserleşmeye doğru giden süreçte TRAIL ve DR4 ölüm reseptöründeki azalma ile buna karşılık özellikle DcR1 ve DcR2 dekoy reseptörlerindeki artış, apoptozise direnç oluşturma etkileri nedeniyle bu moleküllerin kanserleşme sürecine katkıda bulunduğunu düşündürmektedir.

Endometrium kanserinin prekanseröz lezyonları olarak değerlendirilen endometrial hiperplazi dokularında TRAIL/TRAIL reseptör ekspresyon profili değerlendirildiğinde oluşturulan grafikte DR4 ekspresyonu en yüksek olarak belirlenirken, istatistiksel olarak değerlendirildiğinde DR4'ün TRAIL ve DcR1' e göre anlamlı derecede yüksek olmadığı saptanmıştır. En düşük oranda sentez edilenler ise DR5 ve DcR2 reseptörleri olmuştur. TRAIL ligand ve reseptör ekspresyonları arasındaki olası ilişkileri belirlemek için Spearman Rho Korelasyon testi uygulanmış ve DR4 ölüm reseptörü ve DcR2 dekoy reseptörü ($p<0,01$) ve TRAIL ligandı ve DcR2 dekoy reseptörü arasında pozitif korelasyonlar ($p<0,05$) belirlenmiştir. Skorlamaya dayalı olarak oluşturulan grafikler incelendiğinde hiperplazide karşılaşılan ekspresyon profili ile endometrioid tip adenokarsinomada karşılaşılan profil büyük bir benzerlik göstermektedir. Bu nedenle endometrioid tip adenokarsinoma ile endometrial hiperplazi olgularının TRAIL ligand ve reseptör ekspresyon profilleri istatistiksel olarak da karşılaştırılmıştır. Gruplar arasındaki istatiksel fark Mann-Whitney U test ile değerlendirilmiştir. İki grup arasında istatistiksel olarak anlamlı bir fark gösterilememiştir. Çalışmaya dahil ettiğimiz karsinoma ve hiperlazi grupları arasında TRAIL/TRAIL reseptör ekspresyon profili açısından anlamlı bir fark bulunamamasının altında yatması muhtemel iki neden vardır. Birincisi, hiperplazi grubunda yer alan alt tiplerin sayısal olarak dağılımı sonucu etkilemiş olabilir. 27 endometrial hiperplazili hastanın 1 tanesinde basit atipisiz hiperplazi (% 3,7) varken, 7'sinde kompleks atipisiz hiperplazi (% 25,9) ve 19'unda da kompleks atipili hiperplazi (% 70,4) söz konusudur. TRAIL ligand ve TRAIL reseptör ekspresyonları değerlendirilen hastaların % 70'lik bir dilimini kompleks atipili hiperplazinin oluşturması, elde edilen profilin daha ziyade kompleks atipik hiperplazi profilini yansıttığını düşündürmektedir. Buna ek olarak, endometrial hiperplaziler Tip 1 olarak adlandırılan endometrioid tip adenokarsinomaya doğru ilerleyebilmeleri nedeni ile oldukça önem taşımaktadır. Endometrial hiperplazinin karsinomaya ilerleme riski sitolojik atipinin varlığına ve ağırlığına bağlıdır. Kanser progresyon riski basit atipisiz hiperplazide 1-kat, kompleks atipisiz hiperplazide 3-kat, basit atipik hiperplazide 8-kat ve kompleks atipik hiperplazi ise 29-kat artmaktadır. Buradan yola çıkarak üzerinde çalıştığımız olgularda toplamda endometrioid tip adenokarsinomaya dönüşme riski yüksek kompleks atipik hiperplazide de endometrioid tip adenokarsinomada karşılaşılan TRAIL/TRAIL reseptör ekspresyon profili ile benzer olduğu gözlenmektedir. Bu sonuç hastalarda hiperplaziden karsinomaya geçiş riskinin değerlendirilmesinde TRAIL ligand ve reseptörlerinin ekspresyon durumunun önemli bir parametre olarak kullanılabileceğini düşündürmektedir.

Normal endometrium ile endometrial hiperplazi TRAIL ligand ve reseptör ekspresyonları da karşılaştırılmış ve iki grup arasındaki istatiksel fark Mann-Whitney U test ile değerlendirilmiştir. Sonuç olarak hiperplazi dokularında normal endometriuma göre DR4 ($p=0,001$) ve DR5 ($p=0,001$) ölüm reseptörleri ile DcR1 ($p=0,008$) dekoy reseptör ekpresyonları farklı olarak bulunmuştur. Normal endometriumdan hiperplaziye geçişte DR4 ölüm reseptöründe azalma, DR5 ölüm reseptörü ile DcR1 dekoy reseptöründe ise artış belirlenmiştir. Bu değişimler de daha önce normal dokudan kanserleşmeye doğru giden süreçte belirlenen değişimlerle paralel olarak belirlenmiştir. Bu durum normal-hiperplazi-karsinoma şeklindeki kanser gelişim sürecinde özellikle DR4 ekpresyonundaki azalma ve DR5-DcR1

ekpresyonlarındaki artışların fonksiyonel bir role sahip olabileceklerini düşündürmektedir.

Endometrioid tip adenokarsinomalı olguların FIGO evreleme sistemine göre 40 tanesi Evre IA (% 40), 28 tanesi Evre IB (% 28), 6 tanesi Evre IIB (% 6), 8 tanesi Evre IIIA (% 8), 2 tanesi Evre IIIB (%2), 14 tanesi Evre IIIC (% 14) ve 1 tanesi Evre IVB (% 1)'de yer almaktadır. TRAIL ligand ve reseptör sentez profili ile hastalığın klinik evresi arasında bir ilişkinin olup olmadığının aydınlatılabilmesi için Spearman rho korelasyon testi uygulanmıştır. Hastaların klinik evresi ile TRAIL/TRAIL reseptör ekspresyon profili arasında istatistiksel olarak anlamlı bir ilişki saptanamamıştır.

Endometriod tip adenokarsinoma hastalarına ait tümörlerin 63 tanesi Grade 1 (% 63), 26 tanesi Grade 2 (% 26) ve 11 tanesi Grade 3 (% 11)'e aittir. TRAIL ligand ve reseptör sentez profili ile olguların grade'leri arasında bir ilişkinin açıklanabilmesi için Spearman rho korelasyon testi uygulanmıştır. Hastaların gradeleri ile TRAIL/TRAIL reseptör ekspresyon profili arasında istatistiksel olarak anlamlı bir ilişki belirlenememiştir.

Endometrial karsinomada değerlendirilen prognostik parametrelerden biri de myometrial invazyon derinliğidir. Endometrioid tip adenokarsinomalı hastaların 9 tanesinde invazyon yok iken (% 9), 45 tanesinde invazyon derinliği ≤ ½ (% 45) ve 46 tanesinde invazyon derinliği > ½ (% 46)'dır. TRAIL ligand ve reseptör sentez profili ile olguların myometrial invazyon derinliği arasında bir ilişkinin açıklanabilmesi için Spearman rho korelasyon testi uygulandı. Hastalardaki mevcut myometrial invazyon derinliği ile TRAIL/TRAIL reseptör ekspresyon profili arasında istatistiksel olarak anlamlı bir ilişki saptanamamıştır.

Çalışmamızda, TRAIL ligand ve reseptörlerinin ekspresyon profillerinin yanı sıra normal endometrium, hiperplazi ve karsinoma şeklindeki gelişim basamaklarında apoptozis durumunun belirlenebilmesi için TUNEL metodu kullanılmıştır. Yapılan analizler sonucunda normal-hiperplazi-karsinoma sıralamasında karsinomaya doğru gidişte apoptozis yüzdesinde istatistiksel olarak anlamlı bir artış tespit edildi. TRAIL ve reseptörleri ile apoptozis arasındaki ilişkinin açıklanabilmesi için spearman rho korelasyon testi uygulandı; ancak herhangi bir korelasyon bulunamadı. Yapmış olduğumuz çalışmada elde edilen bulgulara göre normal doku ile kanser dokusu karşılaştırıldığında, kanser dokusunda DR4 ölüm reseptöründe azalma ile DR5 ölüm reseptörü ve DcR1 dekoy reseptör ekspresyonundaki artışlar daha çok TRAIL aracılı apoptozise karşı dirençlilik ile ilişkili bir durumu ön plana çıkarıyor gibi görünmektedir. Ancak burada normal dokuya göre kanser dokusunda apoptozis yüzdesinde önemli bir artış karşımıza çıkmaktadır.

SONUÇLAR

Yapmış olduğumuz çalışmada endometrium kanserinde TRAIL ligand ve reseptör ekspresyon profillerinin belirlenebilmesi amacıyla normal, endometrial hiperplazi ve endometrial karsinoma doku örneklerinde TRAIL ligand ve reseptörlerine spesifik antikorlar kullanılarak immunohistokimyasal analizler gerçekleştirilmiştir. TRAIL ligand ve reseptörlerinin normal, hiperplazi ve karsinoma örneklerinde sergilemiş oldukları ekspresyon profilleri ve bu dokularda karşılaşılan apoptozis oranları, bu moleküllerin hücrelerdeki mevcut ölüm ve yaşam dengesinin bozulmasını sağlayarak endometrium kanser gelişim sürecine katkı sağlayabileceğini düşündürmektedir.

Bu çalışmada ayrıca, TRAIL ölümcül ligandı ve reseptör ekspresyonları ile hastalığın klinik evresi, grade ve myometrial invazyon gibi prognostik faktörlerin ilişkisi araştırılmış, istatistiksel olarak anlamlı bir ilişki kurulamamıştır. Ancak, elde edilen bulguların farklı prognostik veriler ile de değerlendirilmesi gerekmektedir. Yapmış olduğumuz çalışmada normal, hiperplazi ve kanser geçiş basamaklarında özellikle kansere dönüşme riski yüksek olan kompleks atipili hiperplazi ve karsinoma dokularındaki TRAIL ve reseptör ekspresyon profilinin benzer olması, diğer taraftan normal dokudan farklı bir profil sergilemesi hastalığın seyrinin takibi ve risk değerlendirilmesi açısından bu moleküllerin prognostik birer belirteç olarak kullanılabileceğine işaret etmektedir.

Tüm bunlara ek olarak endometrium kanserinde TRAIL ve reseptörlerinin ekspresyon profillerinin belirlenmesinin, kanserli hücrelerin apoptozise götürülmesini hedefleyen ve TRAIL transferini kapsayan gen tedavi çalışmalarının başarısını belirlemede ve gerekli durumlarda TRAIL dirençliliğinin üstesinden gelmede faydalı olacaktır. Endometrial kanser dokularında özellikle DR4 ve DR5 ölüm reseptörlerinin varlığıda TRAIL aracılı gen tedavi uygulamalarında olumlu yanıt alınabilmesini sağlaması açısından büyük önem taşımaktadır.

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ÖZGEÇMİŞ

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NF-κB targeting by way of IKK inhibition sensitizes lung cancer cells to adenovirus delivery of TRAIL

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Abstract

Background: Lung cancer causes the highest rate of cancer-related deaths both in men and women. As many current treatment modalities are inadequate in increasing patient survival, new therapeutic strategies are required. TNF-related apoptosis-inducing ligand (TRAIL) selectively induces apoptosis in tumor cells but not in normal cells, prompting its current evaluation in a number of clinical trials. The successful therapeutic employment of TRAIL is restricted by the fact that many tumor cells are resistant to TRAIL. The goal of the present study was to test a novel combinatorial gene therapy modality involving adenoviral delivery of TRAIL (Ad5hTRAIL) and IKK inhibition (AdIKKβKA) to overcome TRAIL resistance in lung cancer cells.

Methods: Fluorescent microscopy and flow cytometry were used to detect optimum doses of adenovirus vectors to transduce lung cancer cells. Cell viability was assessed via a live/dead cell viability assay. Luciferase assays were employed to monitor cellular NF-κB activity. Apoptosis was confirmed using Annexin V binding.

Results: Neither Ad5hTRAIL nor AdIKKβKA infection alone induced apoptosis in A549 lung cancer cells, but the combined use of Ad5hTRAIL and AdIKKβKA significantly increased the amount of A549 apoptosis. Luciferase assays demonstrated that both endogenous and TRAIL-induced NF-κB activity was down-regulated by AdIKKβKA expression.

Conclusions: Combination treatment with Ad5hTRAIL and AdIKKβKA induced significant apoptosis of TRAIL-resistant A549 cells, suggesting that dual gene therapy strategy involving exogenous TRAIL gene expression with concurrent IKK inhibition may be a promising novel gene therapy modality to treat lung cancer.

Backround

Lung cancer is the leading cause of cancer mortality in the world (31% for men and 26% for women of all cancer deaths) [1]. Despite the use of conventional multimodal treatment methods (chemotherapy, radiation, and surgery), the overall survival rate from lung cancer has improved little, with <15% of patients surviving >5 years [2]. Consequently, new therapeutic strategies, such as gene therapy, are being tested in preclinical and clinical settings. Knowing that apoptosis is a key mechanism in the regulation of tissue homeostasis, several members of the tumor necrosis factor (TNF) superfamily have been implicated in the process. TNF-related apoptosis-inducing ligand (TRAIL), also known as Apo2L, was originally identified through its homology to TNF, FasL, and other members of the TNF superfamily [3,4]. Like most other members of the TNF superfamily of ligands, TRAIL is primarily expressed as a type II membrane protein of 33-35 kD [5]. To date, four human membrane-bound receptors for TRAIL have been identified: DR4/TRAIL-R1, DR5/TRAIL-R2/KILLER, TRID/DcR1/TRAIL-R3, and DcR2/TRAIL-R4. Two of the membrane receptors, DR4 and DR5, contain the essential cytoplasmic death domain through which TRAIL can transmit an apoptotic signal. DcR1 and DcR2 can also bind TRAIL, but they appear to act as antagonistic receptors because they lack a functional death domain [6-9].

There are several reasons why TRAIL is of interest for people working on cancer gene therapy. TRAIL is unique in that it selectively induces apoptosis in tumor and transformed cells, but does not harm normal cells [10,11]. In addition, apoptosis induction in response to

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most DNA-damaging drugs usually requires functional tumor supressor p53 gene [12]. Because of the inactivation of p53 in more than 50% of human cancers during tumorigenesis, the tumors eventually display resistance to both radiotherapy and chemotherapy. TRAIL, however, can induce p53-independent apoptosis of cancer cells [13]. Despite this fact, a significant proportion of tumor cells display TRAIL resistance by a mechanism that is not yet fully understood [14,15]. Resistance to TRAIL-induced apoptosis, both in normal and cancer cells, was initially considered to be due to DcR1 and/or DcR2 expression, which compete with DR4 and DR5 for binding to TRAIL [6,16]. Apart from TRAIL receptor composition, [17,18] there are a number of other possible reasons why some cancer cells exhibit TRAIL resistance. For example, the presence of intracellular apoptosis inhibitory proteins (Bcl-xL, c-FLIP, cIAP etc.) or the loss of Bax and Bak function may lead to a TRAIL-resistant phenotype [14,19]. Interestingly, the engagement of DR4, DR5, and DcR2 can activate the NF-κB pathway [20,21], and high levels of endogenous NF-κB activity interfere with TRAIL-induced apoptosis. Thus, targeting the NF-κB signaling pathway may help sensitize cancer cells to TRAIL. In this study, a complementary gene therapy modality using adenovirus-mediated delivery of an IKKβKA mutant (AdIKKβKA) was deployed to test the extent to which NF-κB inhibition sensitized lung cancer cells to TRAIL (Ad5hTRAIL).

Methods

Adenovirus Preparation

Recombinant adenoviral vectors, AdEGFP [22], Ad5h-TRAIL [23], AdIKKβKA [24], AdNFκBLuc [25], and AdCMVLacZ [26,27], were amplified in 293 cells and purified by cesium chloride gradient. After vector purification, adenoviral vectors were kept at -80°C in 10 m*M* Tris containing 20% glycerol. The titers of purified adenoviral stocks were measured to be 10^{13} DNA particles/ml. AdIKKβKA encodes the dominant negative mutant form (K44A) of IKKβ and forms inactive IKK complex so that IKKβ does not phosphorylate IkB. IkBαSR produces dominant negative mutant form (S32A/S36A) of IkBα. Thus, the IKK complex cannot phosphorylate mutant IkBα from S32 and S36 residues. By doing so NF-κB is always sequestered in cytoplasm. Both mutant proteins interfere with NF-κB signaling at different levels of the signaling cascade.

Cell Culture

The human non-small cell lung carcinoma cell line A549 was obtained from American Type Culture Collection. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2.2 g/l sodium bicarbonate, 1 mM L-glutamine, and 1% penicillin-streptomycin-amphoterisine mixture (PSA) using Thermo SteriCult incubators. The study was carried out in accordance with Declaration of Helsinki and approved by the Akdeniz University Committee on Ethics.

Adenoviral Infection of Lung Cancer Cells

Cells were cultured and permitted to adhere for at least 24 hr before adding adenovirus vectors. Before the infection, lung cancer cells were washed with PBS, and then infected with vectors at increasing multiplicity of infection (MOI). Cells were first kept at 37°C in RPMI 1640 medium without FBS for 2 h. An equal volume of RPMI 1640 supplemented with 20% FBS was then added to cells. To measure transduction efficiency, the percentage of $EGFP^+$ cells was determined by using fluorescent microscopy and flow cytometry 48 h after infection. The cell viability was assessed using Propidium iodide exclusion technique.

Cell Viability Assay

Live/Dead Cellular Viability/Cytotoxicity Kit (Molecular Probes; Eugene, OR) was used to discriminate live cells from dead cells. This assay is based on the use of Calsein AM and Ethidium homodimer-1 (EthD-1). Calsein AM is a fluorogenic substrate for intracellular calsein esterase. It is modified to a green fluorescent compound (calsein) by active esterase in live cells with intact membranes. In addition, live cells do not allow EthD-1, a red fluorescent nucleic acid stain, to enter. However, cells with damaged membrane uptake the dye and stain positive. Cellular viability assays were conducted 35 h following the infections.

NF-κB Transcription Induction Experiments

A549 cells were infected with AdNFkBLuc construct at an MOI of 5000 DNA particles/cell to determine the NF-κB activation status. AdNFkBLuc vector carries four tandem copies of the NF-κB binding consensus sequence fused to a TATA-like promoter from the HSVTK gene. This vector has also a Luciferase reporter gene. Luciferase assays were conducted 30 h following the infection using the Luciferase assay system with Reporter Lysis Buffer as described by the manufacturer (Promega, Inc.). Bradford assay was performed to measure the protein concentration in each sample and these values have subsequently been used to normalize Relative Light Units (RLU) against the protein concentration.

Flow Cytometry and Western Blotting

Flow cytometry assays were conducted as described previously [28]. Monoclonal antibody to TRAIL (human) (cat. no. ALX-804-296-C100; Alexis Biochemicals) was

used followed by polyclonal antibody to mouse IgG1 (R-PE) (cat. no. ALX-211-201-C050; Alexis Biochemicals) to reveal TRAIL expression on the cell surface. For Western Blotting, protein extracts were prepared 48 hours following the infection. Then, 10 μg of A549 cell line extract was loaded in each lane and IKKβKA protein expression was detected using an anti-HA peroxidase antibody (Roche Molecular Diagnostic, Indianapolis, Indiana, US, Cat. No.11667475001). GAPDH expression was detected using a GADPH antibody (BIODESIGN International, Maine, US, Cat No. H86504).

Confirmation of apoptosis induction by Annexin V staining using flow cytometry

FITC-conjugated human Annexin V (ALX-209-250-T100) was used to quantitate the number of apoptotic cells using flow cytometry. Annexin V staining procedure was performed according to manufacturer's protocols (Alexis Biochemicals).

Caspase Activity Assays

It is well established that carboxyfluorescein-labeled caspase inhibitors can irreversibly bind to active caspases.

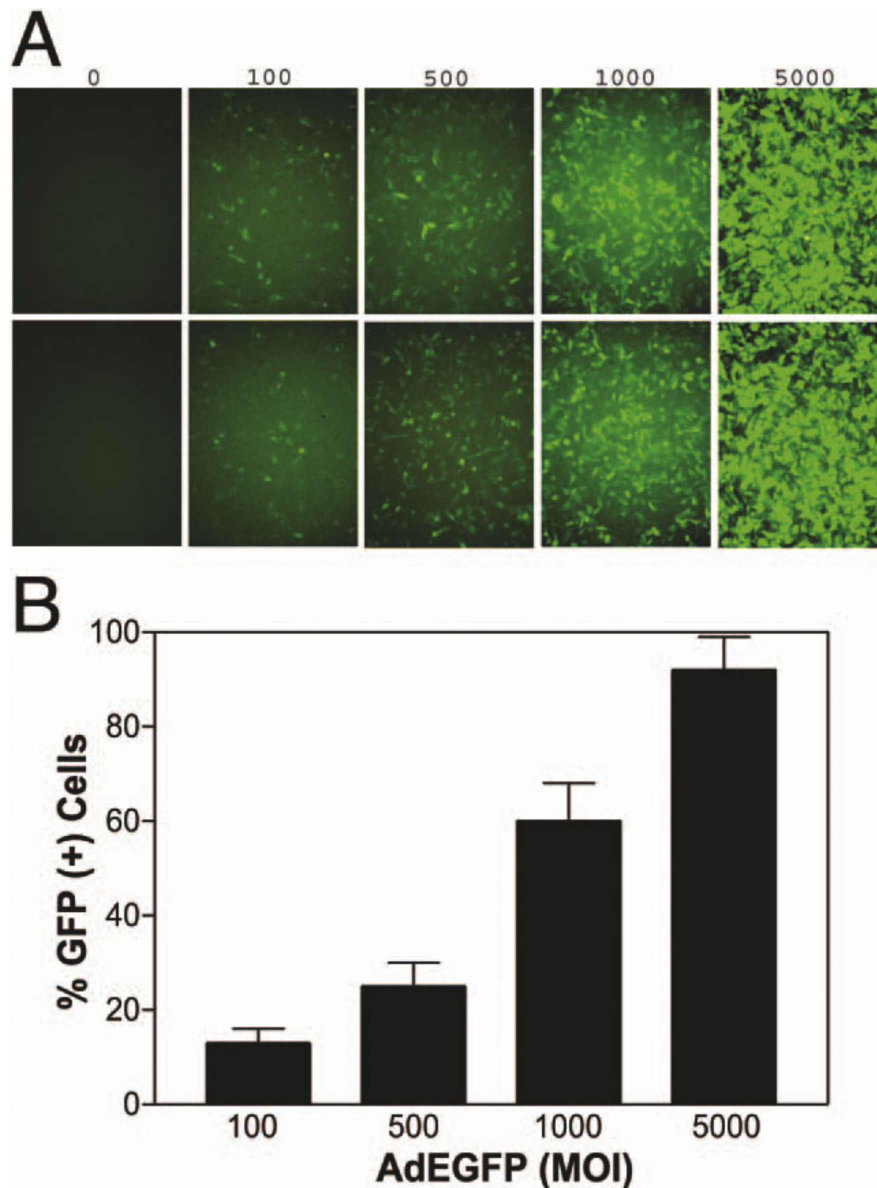

Figure 1 Efficient transduction of A549 cells with recombinant adenovirus. A549 cells were infected with increasing MOI of adenovirus encoding the EGFP reporter gene for 48 h. EGFP positive cells were detected by fluorescent microscopy (Panel A), and then analyzed by flow cytometry (Panel B). Viral doses applied as MOI values in DNA particles/cell are indicated.

The caspase inhibitor substrates were designed to be not only specific for the active state of the enzyme and also it is isoform specific. CaspaTag Caspase Activity Kits were deployed to selectively monitor caspase activation following infection with gene therapy vectors. FAM-DEVD-FMK (S7301) was used to measure caspase 3 activation, and then distinguished caspase positive cells from caspase negative cells by immune fluorescence microscopy.

Results

Efficient adenoviral transduction of A549 lung cancer cells

The efficacy of recombinant adenoviral vector transduction of A549 lung cancer cells was revealed using an AdEGFP vector to determine the optimum dose of adenovirus needed to conduct gene delivery, which is mainly influenced by viral preparations. While fluorescent microscopy was used to follow protein expression, the transduction efficiency was quantitatively analyzed using flow cytometry 48 h following the infection. Almost 100% of the cells were efficiently transduced with AdEGFP at MOI of 5,000 DNA particles/cell 48 h after infection (Figure 1).

A549 lung cancer cells are resistant to adenoviral delivery of hTRAIL or IKKβKA expression

Despite the fact that TRAIL can potently induce tumor cell apoptosis, TRAIL resistance observed in some cancer cells critically challenges the use of TRAIL as a gene therapy agent. To determine the extent to which A549 lung cancer cells were susceptible to TRAIL, we infected A549 cells with an adenovirus vectors encoding hTRAIL (Ad5hTRAIL) or LacZ (AdCMVLacZ, negative control) at increasing doses and measured cellular viability following the infection. As expected, AdCMVLacZ infection alone did not reduce the number of viable cells significantly (data not shown). A549 lung cancer cells were also completely resistant to cytotoxic effects of hTRAIL, despite the high doses of Ad5hTRAIL (MOI of 10,000 DNA particles/cell) used for the infection (Figure 2, upper panels).

Increased IKK activity [29,30] and/or NF-κB activity [22] is a major regulatory obstacle against death ligand-induced cytotoxicity in various tumors. Consequently, cell survival mediated through the effect of IKK inhibition, and thereby NF-kB down-regulation, was tested after A549 infection with AdIKKβKA. As shown in Figure 2 (lower panels), no decrease in cell viability was observed even at MOI of 10,000 DNA particles/cell of AdIKKβKA vector. These results suggested that IKK inhibition alone does not affect the viability of A549 lung cancer cells.

To rule out the possibility that the lack of any cytotoxic effect was due to little/no TRAIL expression from the vector, flow cytometric analysis was performed on A549 cells infected with Ad5hTRAIL. This assay demonstrated that significant TRAIL overexpression was achieved after

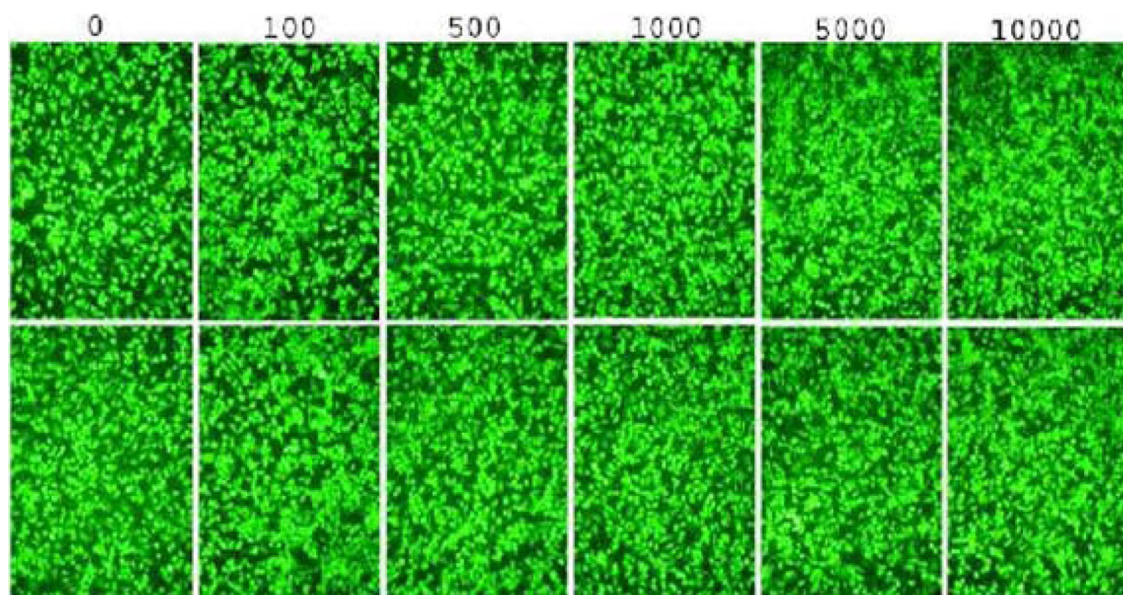

Figure 2 The viability of A549 lung cancer cells is not affected by Ad5hTRAIL or AdIKKβKA infection alone. A549 cells were infected either by Ad5hTRAIL (Upper Panels) or AdIKKβKA virus (Lower Panels) at increasing doses. Molecular Probe's Live and Death Cellular Viability and Toxicity Kit was used to detect viable cells 48 h following the infection, as described in Materials and Methods. The applied viral doses as MOI values in DNA particles/cell are indicated.

A549 infection with Ad5hTRAIL (Figure 3A). Similarly, immunoblot analysis was employed to demonstrate IKKβKA expression. IKKβKA expression was detectable only when cells were infected with AdIKKβKA vector but not with AdCMVLacZ (Figure 3B).

NF-κB blocking via IKK inhibition sensitizes A549 lung cancer cells to TRAIL-induced apoptosis

Previous studies by our group have shown that A549 lung cancer cells can be sensitized to TNF by AdIKKβKA [22,24] or AdIkBαSR [26,31] expression. Since A549 cells are also resistant to TRAIL-induced apoptosis, we tested the extent to which NF-κB inhibition through IKK targetting could sensitize A549 cells to TRAIL. Thus, A549 cells were co-infected with Ad5hTRAIL (5000 particles/cell) and AdIKKβKA at increasing doses, and the percentage of viable cells was measured 48 h after infection. Over 75% cell death was observed when A549 cells were co-infected with Ad5hTRAIL and at least 5000 MOI AdIKKβKA (Figure 4). In contrast, AdCMVLacZ co-infection did not sensitize A549 cells to TRAIL. Together, these findings demonstrate that IKKβKA expression can overcome TRAIL resistance in A549 lung cells. We then tested the extent

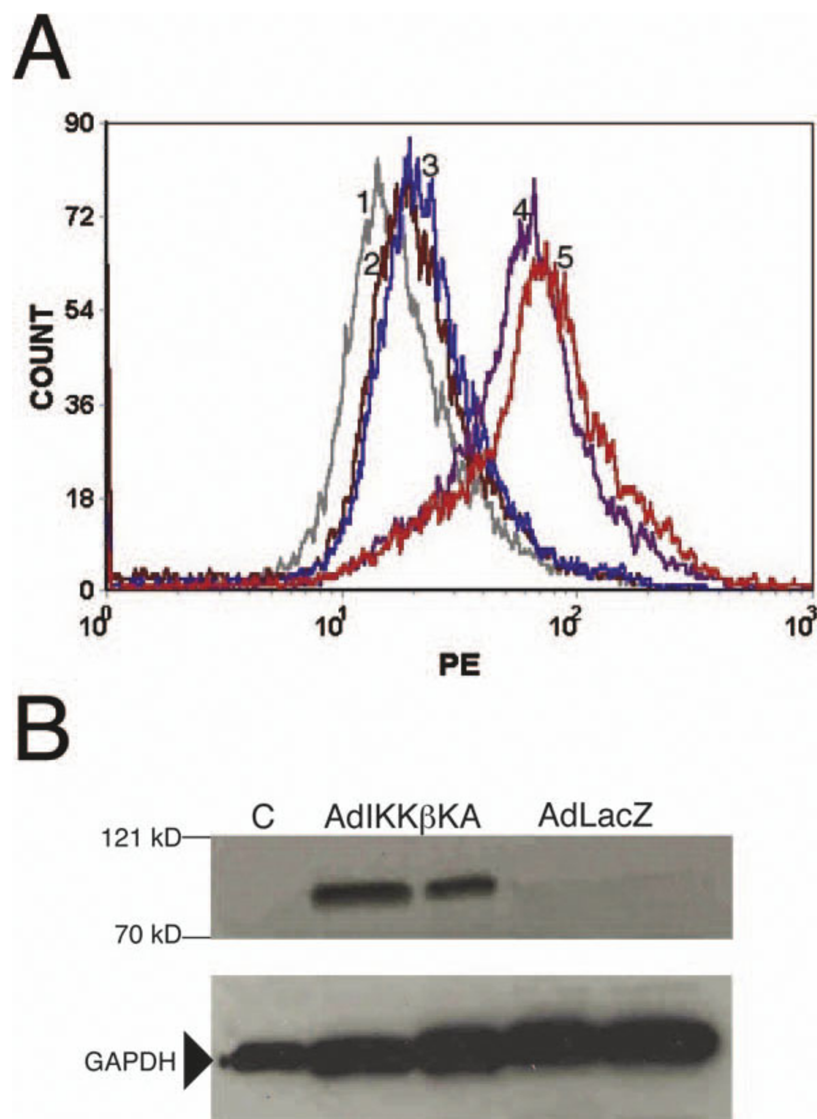

Figure 3 Ad5hTRAIL and AdIKKβKA transductions of lung cancer cells. Panel A represents a flow cytometry analysis of hTRAIL expression in A549 cell line. Conditions for infections are as follows: 1, unstained; 2, uninfected (secondary antibody alone); 3, AdLacZ; 4, Ad5hTRAIL (5,000 MOI); 5, Ad5hTRAIL (10,000 MOI). Panel B shows a Western Blotting indicating IKKβKA expression. Adenoviral constructs used in the infections are indicated above each lane (duplicate independent A549 samples are shown). Molecular standard markers (β galactosidase, 121 kD; and bovine serum albumin, 70 kD) are provided to the left of the blot.

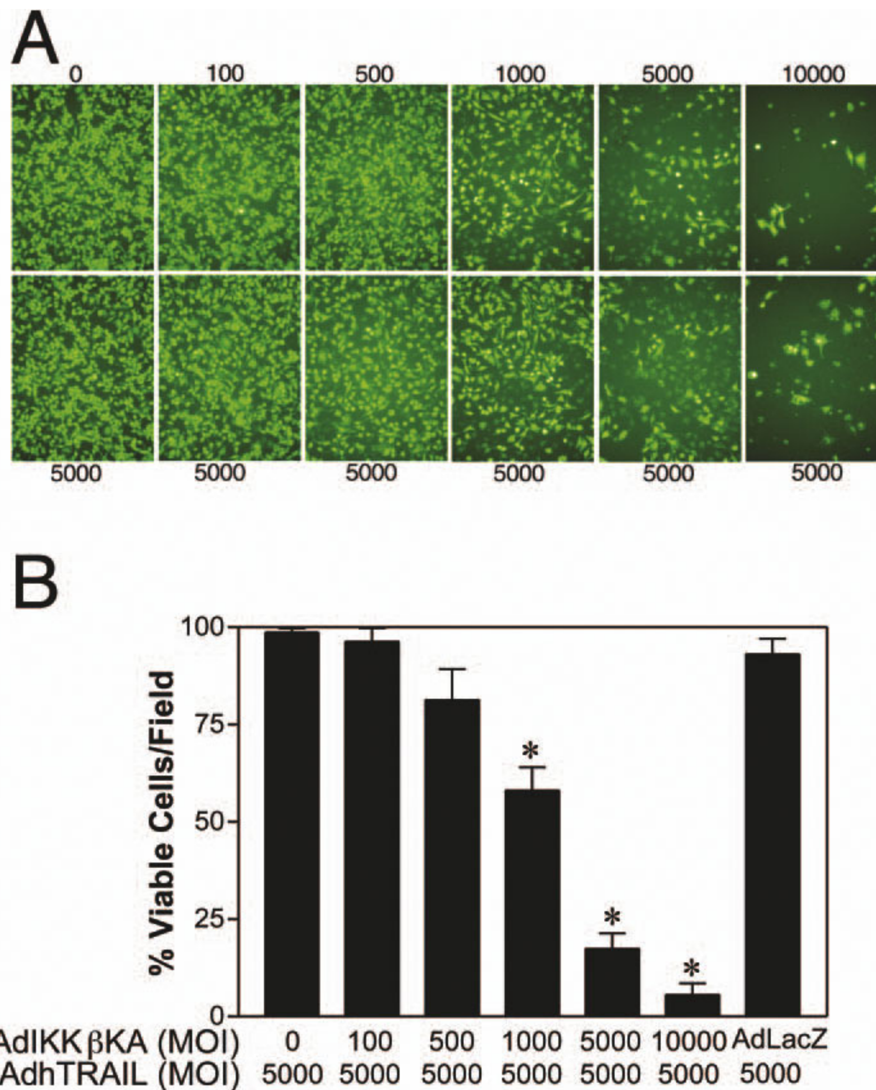

Figure 4 Induction of cell death in A549 cells co-infected with AdIKKβKA and AdhTRAIL. A549 lung cancer cells were co-infected with a constant dose of Ad5hTRAIL (5,000 MOI; as shown below each panel) and increasing doses of AdIKKβKA (as shown above each panel). Cell viability was then measured using Molecular Probe's Live and Death Cellular Viability and Toxicity Kit 48 h following infection. Panel A depicts fluorescent micrographs of such an infection. By comparison, the quantitative analysis of cell viability is provided in Panel B. Values represent the mean (± SEM) of three different experiments (n = 6). *$p < 0.05$.

to which AdIkBαSR could substitute for the AdIKKβKA vector in sensitizing A549 cells to Ad5hTRAIL. NF-κB inhibition via AdIkBαSR infection also resulted in some degree of cell death from TRAIL, but the degree of sensitization was less than that of AdIKKβKA delivery (data not shown) suggesting AdIKKβKA inhibition of NF-κB inhibition is more efficient.

Endogenous NF-κB activity in A549 cells is upregulated after Ad5hTRAIL infection but down regulated with IKKβKA expression

Intracellular NF-κB activation is crucial for a variety of cellular functions. With respect to death ligand-induced apoptosis, high levels of NF-κB activity are associated with resistance in cancer cells [22]. In addition, signaling through DR4, DR5 [15,20], and DcR2 [8] can activate NF-κB. Thus, determining the endogenous and TRAIL-induced NF-κB activity of cancer cells before initiation of TRAIL-based therapy is important. To evaluate the extent of NF-κB activation in A549 cells, we used a recombinant adenovirus vector encoding an NF-κB driven Luciferase reporter gene (AdNFkBLuc). Luciferase expression was measured 30 h after infection. As shown in Figure 5, significant NF-κB activation was achieved after Ad5hTRAIL infection. Triple infection with AdNFkBLuc, Ad5hTRAIL and AdIKKβKA or AdCMVLacZ was then performed to

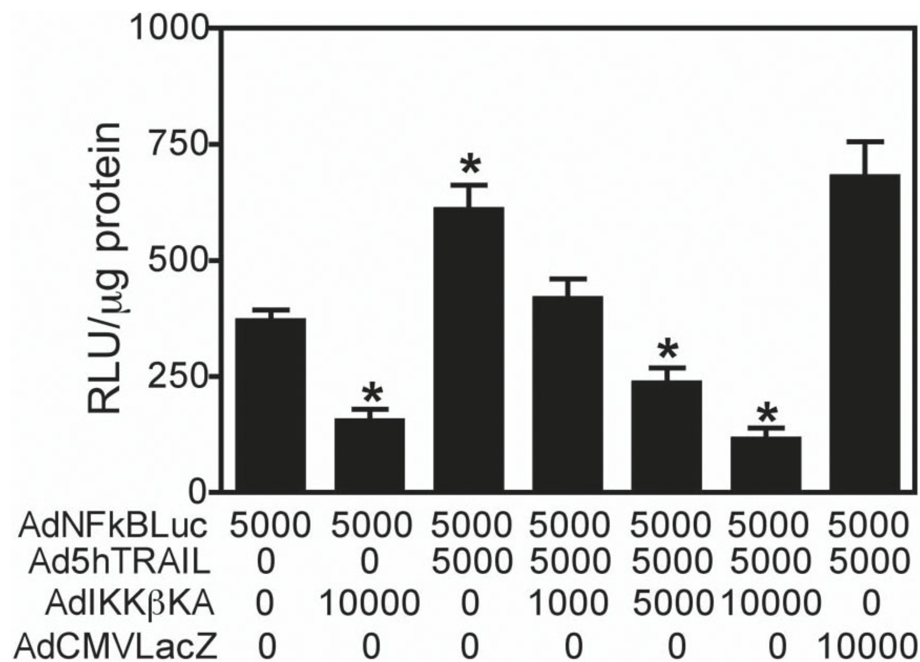

Figure 5 NF-κB activity is upregulated with Ad5hTRAIL infection but down-regulated with AdIKKβKA in A549 cells. A549 cells were simultaneously infected with AdNFkBLuc, Ad5hTRAIL, and increasing doses of AdIKKβKA construct for 30 h. As a negative control, AdCMVLacZ infection was utilized. The different constructs used in the infection and the MOI values represented in DNA particles/cell are listed on the X-axis. Luciferase activity expressed as Relative Light Units per microgram protein is depicted on the Y-axis, where the values represent the mean (± SEM) of six independent data points (n = 6). * $p < 0.05$.

check the extent to which AdIKKβKA reduced NF-κB activation. Both endogenous and TRAIL-induced NF-κB activity was drastically reduced after infection with AdIKKβKA. Conversely, AdCMVLacZ infection did not inhibit endogenous or TRAIL-induced NF-κB activity.

Ad5hTRAIL infection with NF-κB inhibition induces apoptosis in A549 cells

To prove that the mechanism of cell death in A549 cells following Ad5hTRAIL infection during IKK inhibition was apoptotic in nature, Annexin V staining was performed. A549 cells were infected with either Ad5hTRAIL or AdIKKβKA alone, or in combination, and apoptotic cell death was quantified 48 h following infection. There was a minimal increase in Annexin V staining on Ad5hTRAIL or AdIKKβKA infected cells (Figure 6A), but Ad5hTRAIL and AdIKKβKA coinfection resulted in a significant increase in Annexin V staining (Figure 6B). As expected, Ad5hTRAIL and AdCMVLacZ co-infection did not generate such an effect. To further demonstrate that apoptosis is the mechanism of cell death in A549 cell line, caspase activation assays were performed following coinfection of cells with Ad5hTRAIL and AdIKKβKA vectors. There was significantly increased Caspase 3 activity detected only when the A549 cells were infected with Ad5hTRAIL and AdIKKβKA (Figure 7).

Discussion

TRAIL induces apoptosis in a wide range of malignant cells and has been heavily investigated as a potential therapeutic agent for the treatment of many tumors. These expectations were largely based on the selective apoptosis-inducing properties of TRAIL for cancer cells [32-34]. Contrary to these initial expectations, many cancer cell lines were subsequently found to be resistant to TRAIL-induced apoptosis. Consequently, a significant number of studies have been conducted to understand the molecular mechanism of TRAIL resistance in cancer cells, so this barrier could be overcome. In cancer cases where high decoy receptor expression could potentially contribute to the resistance to TRAIL, siRNA approaches have been successfully used to overcome TRAIL resistance in cancer cells, as demonstrated for breast [18], lung [28], and prostate [17] cancer cells.

Based on our previous findings and those by other groups, the NF-κB signaling pathway appeared to be one of the main molecular mechanisms responsible for the generation of TRAIL resistance in cancer cells. Overactive NF-κB activity has been implicated in many aspects of tumor formation and progression, including the inhibition of apoptosis and enhancing the expression of antiapoptotic factors [35]. NF-κB normally resides in the cytoplasm as an inactive complex with an inhibitory

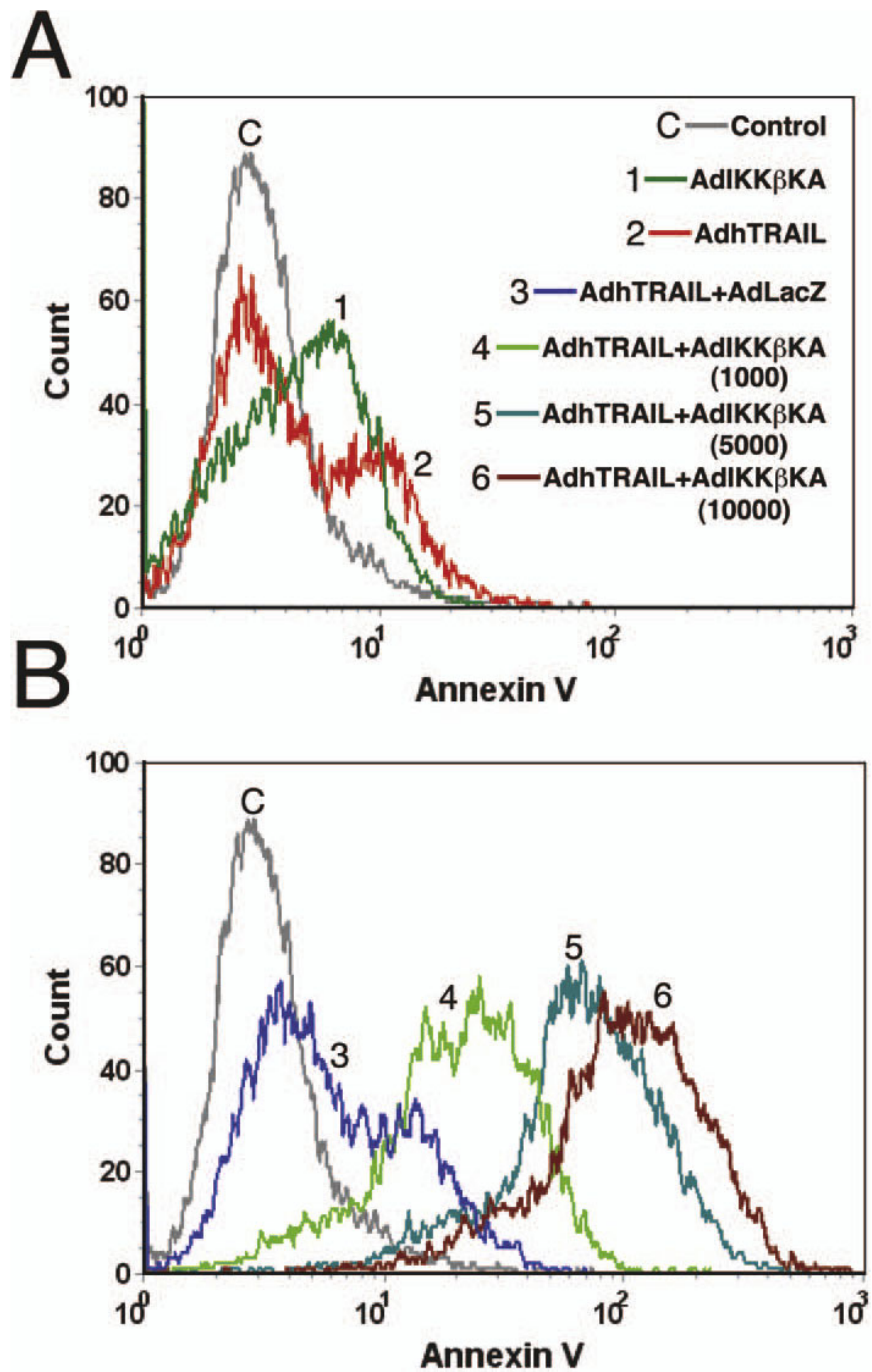

Figure 6 Blocking IKK activity results in TRAIL-induced apoptosis in A549 cells. FITC-conjugated Annexin V and Propidium Iodide (PI) staining were utilized as apoptosis indicators following infection of A549 lung cancer cells with various combinations of adenovirus constructs as stated. 10^4 A549 lung cancer cells were gated for each histogram. Histograms are depicted in two different panels for clarity. AdIKKβKA and Ad5hTRAIL constructs were used in 5,000 DNA particles/cell unless stated otherwise. AdCMVLacZ construct was used at an MOI of 10,000 DNA particles/cell. Uninfected but FITC-Annexin V and PI stained A549 lung cancer cells were used as controls (gray line).

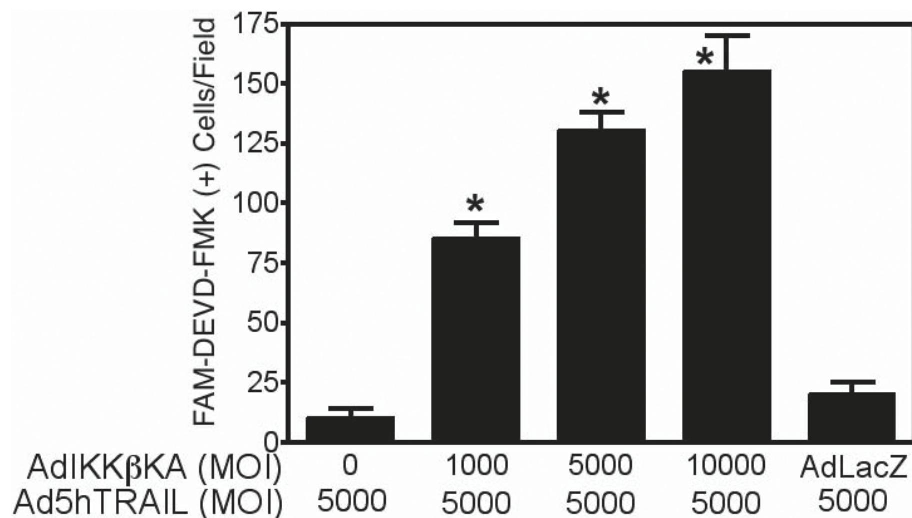

Figure 7 Inhibition of IKK activity induces TRAIL-mediated Caspase 3 activation in lung cancer cells. Caspase activity assays were performed following co-infection of A549 lung cancer cells with Ad5hTRAIL and AdIKKβKA or AdCMVLacZ vectors. MOI and types of viral vectors used in the infection are provided on the X-axis. AdCMVLacZ construct was used at an MOI of 10,000 DNA particles/cell. Caspase activity was assessed under fluorescent microscopy. Data represent the mean of (±SEM) five independent data points. *$p < 0.05$.

IκB subunit. Upon activation, IκB becomes phosphorylated by specific kinases (IκB kinase, IKK), ubiquinated, and then degraded. This inactivation of IκB enables the translocation of NF-κB into the nucleus, where it can bind to the promoter region of many genes and activate their transcription [36]. IKKβ is one of the catalytic domains of the kinase IKK and is essential for NF-κB activation. Thus, inhibition of IKKβ may be a particularly useful strategy to specifically interfere with NF-κB activity [24]. Previously, IKK targeting strategy has been successfully applied to sensitize neuroblastoma [37] and prostate cancer cells [38] to TRAIL. Although, exogenous expression of a dominant negative mutant form of IKKβ sensitized lung cancer cells to TNF by way of NF-κB inhibition, it was unknown whether this approach would similarly sensitize lung cancer cells to TRAIL. Thus, in this study we tested a complementary gene therapy modality involving IKK inhibition to overcome TRAIL resistance. In the present study, we demonstrated that inhibition of the NF-κB signaling pathway, by way of IKKβKA expression, sensitized A549 cells to TRAIL-induced apoptosis. In accordance with this, the recently identificed TRAIL receptor-binding protein, protein arginine methyltransferase 5 (PRMT5) [39], was found to potentiate TRAIL-induced NF-κB activation through IKK leading to induction of several NF-κB target genes. Interestingly, PRMT5 gene silencing sensitized various cancer cells to TRAIL. These data suggest that PRMT5 expression helped to maintain TRAIL resistance through NF-κB activation involving IKK complex in cancer cells.

Conclusions

The IKK complex may be a good target to specifically interfere with NF-κB activation in TRAIL-resistant cancer cells, such that gene therapy strategies involving exogenous TRAIL expression with concurrent inhibition of the NF-κB pathway through IKK modulation of function may extend the therapeutic index of TRAIL for patients with lung cancer.

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Authors' contributions
CA and ADS designed and conducted the study. AB, LD, BK and BY helped with the assays. TSG acted as a consultant and critically reviewed the manuscript. ADS and SS supervised the study. All the authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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RESEARCH ARTICLE | Open Access

TRAIL Death Receptor-4, Decoy Receptor-1 and Decoy Receptor-2 Expression on CD8$^+$ T Cells Correlate with the Disease Severity in Patients with Rheumatoid Arthritis

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Abstract

Background: Rheumatoid Arthritis (RA) is a chronic autoimmune inflammatory disorder. Although the pathogenesis of disease is unclear, it is well known that T cells play a major role in both development and perpetuation of RA through activating macrophages and B cells. Since the lack of TNF-Related Apoptosis Inducing Ligand (TRAIL) expression resulted in defective thymocyte apoptosis leading to an autoimmune disease, we explored evidence for alterations in TRAIL/TRAIL receptor expression on peripheral T lymphocytes in the molecular mechanism of RA development.

Methods: The expression of TRAIL/TRAIL receptors on T cells in 20 RA patients and 12 control individuals were analyzed using flow cytometry. The correlation of TRAIL and its receptor expression profile was compared with clinical RA parameters (RA activity scored as per DAS28) using Spearman Rho Analysis.

Results: While no change was detected in the ratio of CD4$^+$ to CD8$^+$ T cells between controls and RA patient groups, upregulation of TRAIL and its receptors (both death and decoy) was detected on both CD4$^+$ and CD8$^+$ T cells in RA patients compared to control individuals. Death Receptor-4 (DR4) and the decoy receptors DcR1 and DcR2 on CD8$^+$ T cells, but not on CD4$^+$ T cells, were positively correlated with patients' DAS scores.

Conclusions: Our data suggest that TRAIL/TRAIL receptor expression profiles on T cells might be important in revelation of RA pathogenesis.

Background

Rheumatoid arthritis (RA) is a chronic autoimmune disease, which affects the synovial membrane and eventually causes irreversible destruction of tendons, cartilage, and bone [1-3]. It has long been suspected that the inflammatory lesions result from an autoimmune response to joint-specific antigens primarily involving the cells of the immune system [4-7]. Although disease commences with T cells recognizing antigen, this recognition event also drives a chronic inflammatory process involving the activation of macrophages and B cells [8,9]. The chronic inflammation is in turn perpetuated by activation of both CD4$^+$ and CD8$^+$ T cells [10].

Programmed cell death is an apoptotic mechanism by which damaged cells are removed from the body. Engagement of autoreactive T cells by self antigens within the thymus induces deletion of potentially harmful T cells. Defects in apoptosis lead to the persistence of T cells recognizing self antigens which can induce autoimmunity [11,12]. Clonally expanded T cells that have served their functional purpose are also cleared from the system through activation induced cell death (AICD) involving cell surface FasL/Fas receptor interaction [13]. Children with defective Fas-mediated T lymphocyte apoptosis exhibit a disorder known as

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autoimmune lymphoproliferative syndrome [14,15]. Like FasL, TNF has also been involved in AICD [16], although unlike CD95, TNF initiates and exacerbates autoimmune diseases. A third member of TNF superfamily is the TNF Related Apoptosis Inducing Ligand (TRAIL) [17]. In the immune system, TRAIL is expressed on the surface of activated T lymphocytes. Five different receptors interact with TRAIL: TRAIL Receptor-1 (TRAIL-R1/also referred to as DR4), TRAIL Receptor-2 (TRAIL-R2/DR5), TRAIL Receptor-3 (TRAIL-R3/DcR1), TRAIL Receptor-4 (TRAIL-R4/DcR2), and osteoprotegrin (OPG) [18,19]. DR4 and DR5 are the genuine death receptors inducing apoptosis whereas DcR1 and DcR2 function as decoy receptors and physiologically block apoptosis [20,21], which might thus contribute to the pathogenesis of autoimmunity [22,23]. Like CD95L, TRAIL has been reported to be a potent inhibitor of autoimmune arthritis [24]. Unlike TNF and FasL, TRAIL inhibits the activation and proliferation of lymphocytes in vivo, but does not delete them from the system.

It is apparent then that FasL, TNF and the TRAIL/TRAIL receptor system are involved in T cell activation and/or deletion [25]. Accordingly, here we demonstrate the potential usage of TRAIL and the expression profile of its receptors on peripheral T cell subsets as markers to monitor the prognosis of patients with rheumatoid arthritis.

Methods

Clinical Assessment of Patients with Rheumatoid Arthritis

20 RA patients and 12 age-/sex-matched control individuals were enrolled in the study conducted at the Rheumatology Clinic of Akdeniz University Hospitals. RA patients were classified according to the American Rheumatism Association 1987 revised criteria. DAS28-3 scoring (including tender joint counts, swollen joint counts and erythrocyte sedimentation rate-ESR) was used for each RA patient to assess the severity of disease. RA patients previously not treated with disease modifying anti-rheumatic drugs (anti-TNF agents) were admitted to the study. These patients had the history of receiving either non-steroidal anti-inflammatory drugs (NSAIDs) or analgesics prior to analysis.

Collection and analysis of blood samples

Analysis of peripheral blood lymphocytes was performed by direct immunofluorescence flow cytometry using a Coulter EPICS ALTRA XL instrument. The following monoclonal antibodies (mAb) were used: Phycoerythrin (PE) anti-human DR4 (CD261, TRAIL-R1, Cat No: 12-6644-73, eBioscience Inc., San Diego, CA, USA), PE anti-human DR5 (CD262, TRAIL-R2, Cat No: 12-9908-73, eBioscience Inc., San Diego, CA, USA), PE anti-human DcR1 (CD263, TRAIL-R3, TRAILR3, LIT, TRID Cat No: 12-6238-73, eBioscience Inc., San Diego, CA, USA), PE anti-human DcR2 (CD264, TRAIL-R4, TRAILR4, TRUNDD, TNFRSF10 D Cat No: 12-6239-73, eBioscience Inc., San Diego, CA, USA), PE anti-human TRAIL (CD253 Cat No: 12-9927-73, eBioscience Inc., San Diego, CA, USA), Fluorescein isothiocyanate (FITC) anti-human CD4 (L3T4 Cat No: A07750, Beckman Coulter, Immunotech, Marseille, France), FITC anti-human CD8 (Cat No: IM0451U, Beckman Coulter, Immunotech, Marseille, France), PE Mouse IgG1 (κ Isotype Control Cat No: 12-4714-73, eBioscience Inc., San Diego, CA, USA), FITC Mouse IgG1 (κ Isotype Control Cat No: A07795, Beckman Coulter, Immunotech, Marseille, France) and FITC Mouse IgG2a (Isotype Control Cat No: 11-4724-73, eBioscience Inc., San Diego, CA, USA).

Flow Cytometry Procedure

Reaction conditions for FC were as follows: 50 μl of each sample was diluted with 50 μl PBS solution (phosphate buffered saline; 0.01 M sodium phosphate, 0.145 M sodium chloride, Ph 7.2), and stained using 10 μl of either FITC (Fluorescein isothiocyanate) conjugated mouse monoclonal antihuman CD4 antibody or FITC conjugated mouse monoclonal antihuman CD8 antibody. PE (Phycoerythrin) conjugated mouse monoclonal antihuman TRAIL or its receptor antibodies were added subsequently to each tube. Both the activation status of T cells ($CD4^+CD25^+$) and the amount of regulatory T cells ($CD4^+CD25^+FoxP3^+$) present were revealed using CD25 ECD (Beckman Coulter, 6607112) and APC-anti-human Foxp3 (eBioscience, 17-4776-73) antibodies. Tubes were incubated in the dark at room temperature for 20 minutes. Erythrocytes were eliminated from PBL using ammonium chloride lysing solution. After two

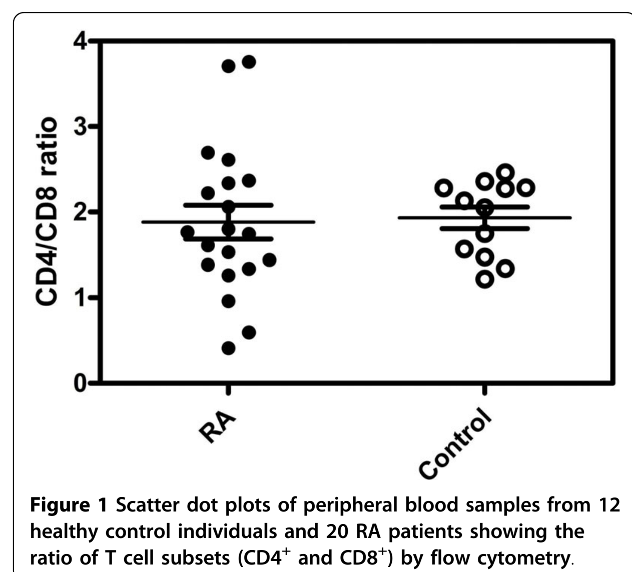

Figure 1 Scatter dot plots of peripheral blood samples from 12 healthy control individuals and 20 RA patients showing the ratio of T cell subsets ($CD4^+$ and $CD8^+$) by flow cytometry.

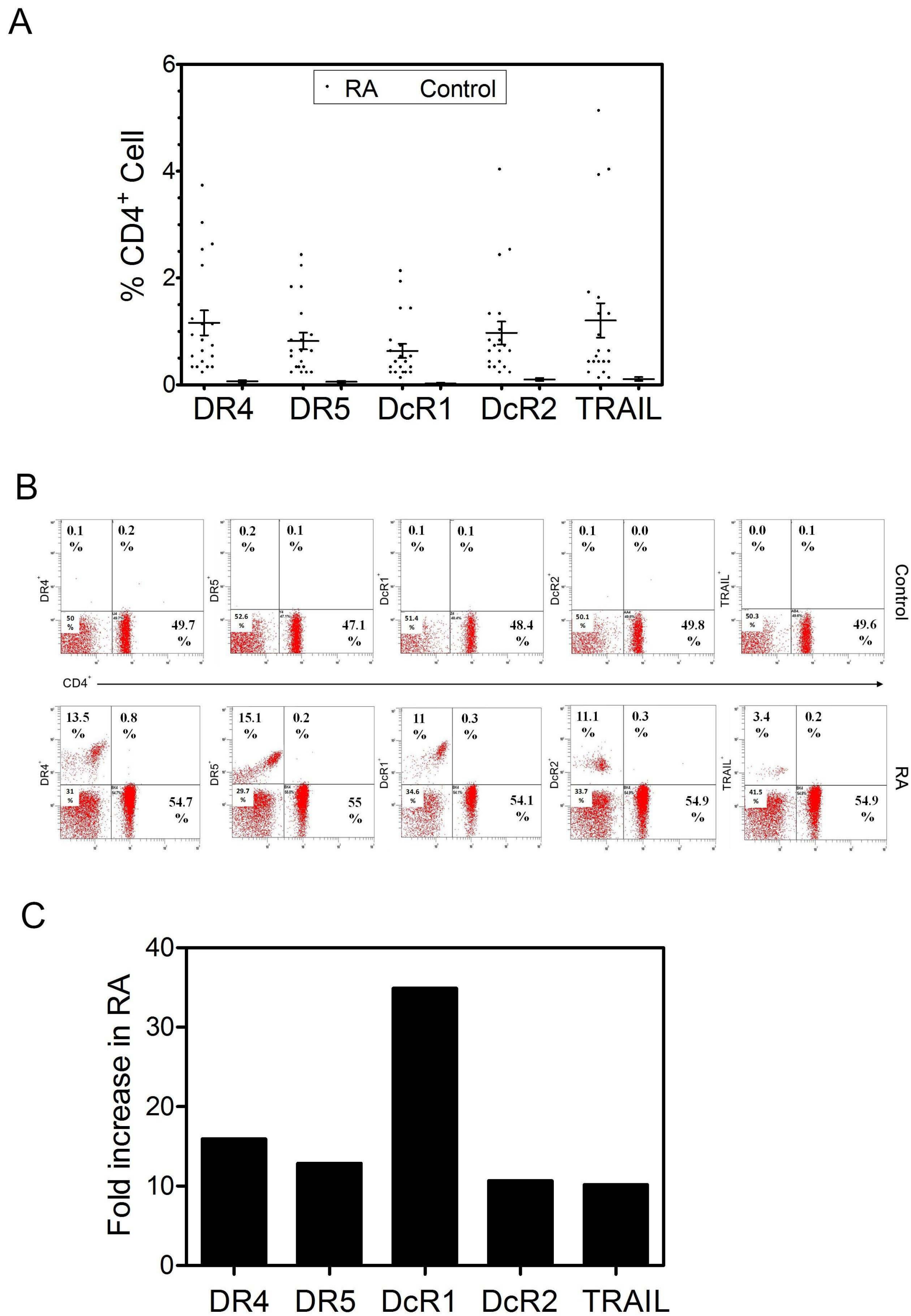

Figure 2 TRAIL and its receptor (both death and decoy) expression profile on CD4+ T cells. A scattered dot plot profile of TRAIL and its receptors is provided in **Panel A**. Solid circles indicate RA patients while dots represent control individuals. Error bars display mean (±) SEM. Representative flow cytometric analyses of a control individual (upper panels) and an RA patient (lower panels) are shown in **Panel B**, while **Panel C** shows the relative increase in TRAIL and its receptors on CD4+ T cells of RA patients compared to controls.

washes with PBS, the cells were resuspended and analyzed by flow cytometry. A calibration based on lymphocyte gating was performed on EPICS Altra XL system using CD45 fluorescence and side scatter parameters prior to analysis. The peripheral blood samples were analyzed within 6 hours so that the gate could still be drawn around lymphocytes. Isotype matched antibodies were included to control for non-specific binding. All results were analyzed using Expo32 Altra software (Beckman-Coulter, Fullerton, CA).

Ethics

Written informed consent relating to the Declaration of Helsinki was obtained from all patients. The study was approved by Akdeniz University Local Committee on Ethics.

Statistical Analysis

The statistical package for the Social Sciences 13.0 software for Windows (SPSS Inc., Chicago, Ill) and GraphPad Prism version 5 (La Jolla, CA, USA) were used to plot the data and perform statistical analyses. In addition, SmartDraw Health Science Edition (San Diego, CA, USA) was used as a graphics software package. Normality tests were conducted using a Shapiro-Wilk method. T cell subsets ($CD4^+$ and $CD8^+$ T cells) in both groups displayed a normal distribution. Thus, a nonparametric unpaired student's T test was used to evaluate $CD4^+/CD8^+$ T cell ratios. On the other hand, a Gaussian distribution was not observed between normal versus RA patients when analyzing TRAIL and TRAIL receptor expression profiles. For this reason, Mann-Whitney U test was used to compare $CD4^+$ versus $CD8^+$ T cell associated TRAIL marker expression in patients. All correlation analyses used Spearman's Rho tests.

Results

Flow Cytometric Analysis of Peripheral Blood T Lymphocytes

Given the heterogeneous nature of rheumatoid arthritis, and evidence for variation in the ratio of $CD4^+/CD8^+$ T cells changes in autoimmune diseases such as Systemic Lupus Erythematosus (SLE) [26-28], we first characterized the composition of peripheral blood T cell subsets in RA patients using flow cytometry. Nonparametric unpaired student's T test was used to detect possible differences in the two T cell subsets between normal versus RA patients. As shown in Figure 1, no difference was detected in the ratio of $CD4^+$ ($p = 0.63$) to $CD8^+$ ($p = 0.22$) T cells between the two groups. We conclude that, unlike Systemic Lupus Erythematosus, disease status does not alter the ratio of $CD4^+$ to $CD8^+$ T cell subsets in RA.

Membrane bound TRAIL and its receptor expression profile on peripheral $CD4^+$ T lymphocytes in RA patients versus control individuals

During the development of RA, $CD4^+$ T cells initiate and regulate several cell-mediated immune processes that cause synovial inflammation and joint destruction in response to activation by antigen presentation. We next examined the $CD4^+$ T lymphocyte associated cell-surface expression profile of TRAIL and its receptors

Table 1 Spearman rho correlation analysis of TRAIL and its receptors present on $CD4^+$ T cells isolated from RA patients

RA-CD4 Spearman's Rho		DR4	DR5	DcR1	DcR2	TRAIL
DR4	correlation Coefficient	1.000	.620(**)	.116	.255	.764(**)
	Sig. (2-tailed)	.	.004	.626	.277	.000
	N	20	20	20	20	20
DR5	correlation Coefficient	.620(**)	1.000	.470(**)	.416	.374
	Sig. (2-tailed)	.004	.	.037	.068	.104
	N	20	20	20	20	20
DcR1	correlation Coefficient	.116	.470(**)	1.000	.639(**)	.086
	Sig. (2-tailed)	.626	.037	.	.002	.718
	N	20	20	20	20	20
DcR2	correlation Coefficient	.255	.416	.639(**)	1.000	.414
	Sig. (2-tailed)	.277	.068	.002	.	.070
	N	20	20	20	20	20
TRAIL	correlation Coefficient	.764(**)	.374	.086	.414	1.000
	Sig. (2-tailed)	.000	.104	.718	.070	.
	N	20	20	20	20	20

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

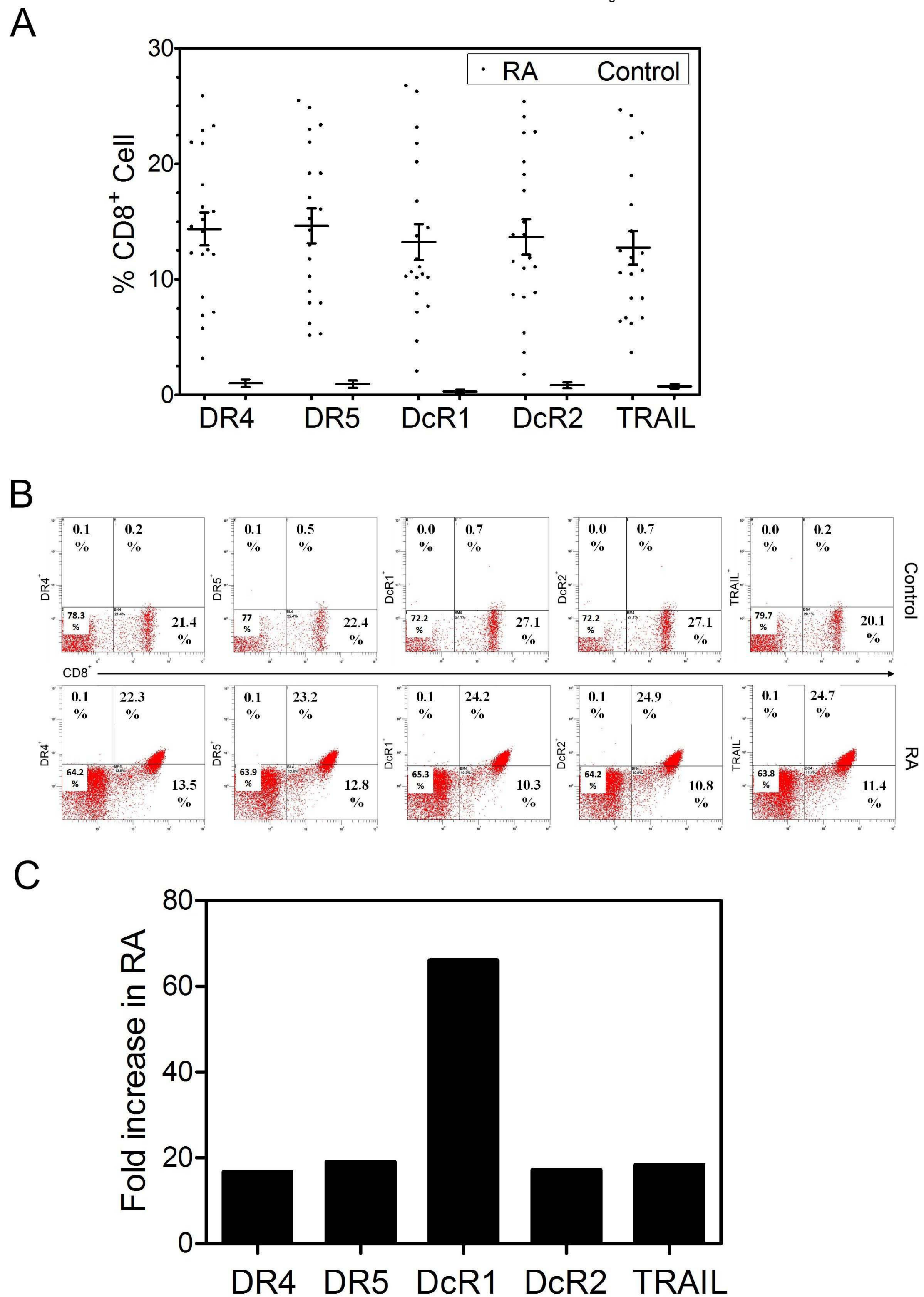

Figure 3 Panel A: CD8$^+$ T cell associated TRAIL and its receptor expression profile. TRAIL and its receptor expression profile of CD8$^+$ T cells gathered from 20 RA patients and 12 healthy controls are shown as a scatter dot plot. Error bars represent mean (±) SEM. **Panel B** shows representative FC for TRAIL and its receptor expression on CD8$^+$ T cells (upper panels show healthy control, lower panels are obtained from an RA patient). **Panel C** shows the relative increase in TRAIL and its receptors on CD8$^+$ T cells on RA cells (vs control).

isolated from either healthy volunteers or RA patients using flow cytometry. The Mann-Whitney U test was used to compare the two groups. As shown in Figure 2A, $CD4^+$ T cells obtained from RA patients exhibited higher levels of expression of TRAIL and its death/decoy receptors compared to cells from control individuals ($p < 0.05$). Representative flow cytometric analyses of $CD4^+$ T cells isolated from a healthy individual and an RA patient are shown in Figure 2B, while Figure 2C indicates the relative increase in marker expression levels. These data show a 35-fold increase in DcR1 expression with a 10-15-fold increase in the other markers.

Using a Spearman Rho correlation test to evaluate correlations between expression of TRAIL and TRAIL receptors on $CD4^+$ T cells in RA patients, we found increased DR4 expression was correlated with an increase in both DR5 and TRAIL (Table 1). In addition, DcR1 and DcR2 expression showed strong correlation ($p < 0.01$).

$CD8^+$ T cell associated TRAIL and its receptor expression profile in RA

We next investigated the expression of TRAIL and its receptors on $CD8^+$ T cells of RA patients and compared with healthy controls, similar to $CD4^+$ T cells, we found significant differences between the two groups as shown in Figure 3A ($p < 0.05$). Representative FC data from a patient and control are shown in Figure 3B, with Figure 3C indicating the relative increase in $CD8^+$ T cell associated TRAIL and its receptor expression. In this case increased DcR1 expression (about 66 fold) was the most marked. A correlation between $CD8^+$ T cell associated TRAIL and receptor expression was investigated using Spearmen Rho Correlation analysis. As shown in Table 2, a correlation among the death and the decoy receptors as well as with TRAIL death ligand expression was observed.

A non-parametric Mann-Whitney U test was administered to investigate statistical differences in expression levels between $CD4^+$ and $CD8^+$ T cell associated TRAIL and its receptors in patients with RA. Our results indicated that expression levels of TRAIL and its receptors were higher on $CD8^+$ T cells (Figure 3A) than on $CD4^+$ T cells (Figure 2A).

$CD8^+$ T cell associated DR4, DcR1 and DcR2 expression levels correlated with DAS28 scores in RA patients

Finally we investigated evidence for a correlation between TRAIL and its receptor expression profile and the severity of disease in RA patients. Interestingly only $CD8^+$ T cell associated (Figure 4) but not $CD4^+$ T cell associated (data not shown) DR4, DcR1 and DcR2 expression levels correlated with DAS28 scores in RA patients.

T cell activation status of newly diagnosed RA patients

CD25 is one commonly used marker for recently activated T cells [29]. In order to document the activation status of T cells in RA patients; another set of flow cytometry assay was conducted. As shown in Table 3, the percentage of $CD4^+CD25^+$ T cells was statistically higher in RA patients compared to control individuals. However, as shown before both activated and regulatory T cells (T_{reg}) can express CD25 marker on the cell surface [30]. To distinguish these two, FoxP3 staining was employed

Table 2 Spearman rho correlation analysis of $CD8^+$ T cell associated TRAIL and its receptors obtained from RA patients

RA-CD8 Spearman's Rho		DR4	DR5	DcR1	DcR2	TRAIL
DR4	correlation Coefficient	1.000	.868(**)	.948(**)	.740(**)	.744(**)
	Sig. (2-tailed)	.	.004	.000	.000	.000
	N	20	20	20	20	20
DR5	correlation Coefficient	.868(**)	1.000	.784(**)	.738(**)	.632(**)
	Sig. (2-tailed)	.000	.	.000	.000	.003
	N	20	20	20	20	20
DcR1	correlation Coefficient	.948(**)	.784(**)	1.000	.773(**)	.689(**)
	Sig. (2-tailed)	.000	.000	.	.000	.001
	N	20	20	20	20	20
DcR2	correlation Coefficient	.740(**)	.738(**)	.773(**)	1.000	.525(*)
	Sig. (2-tailed)	.000	.000	.000	.	.017
	N	20	20	20	20	20
TRAIL	correlation Coefficient	.744(**)	.632(**)	.689(**)	.525(*)	1.000
	Sig. (2-tailed)	.000	.003	.001	.017	.
	N	20	20	20	20	20

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

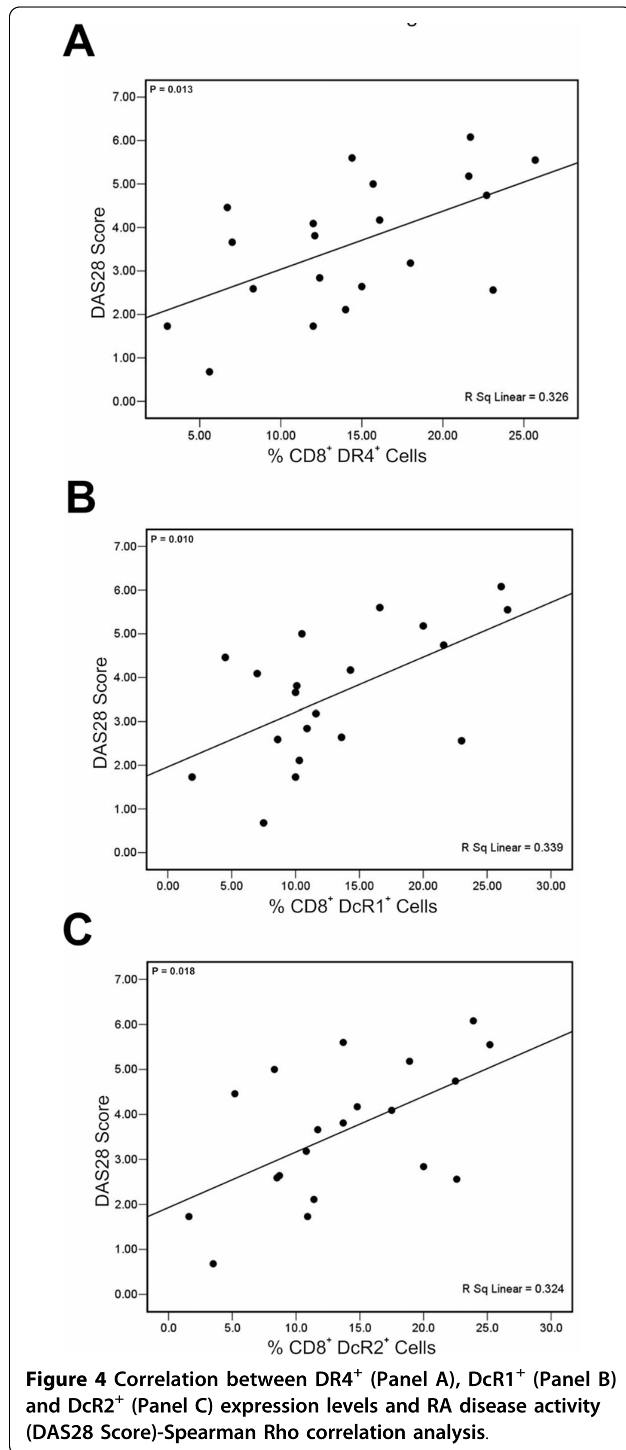

Figure 4 Correlation between DR4⁺ (Panel A), DcR1⁺ (Panel B) and DcR2⁺ (Panel C) expression levels and RA disease activity (DAS28 Score)-Spearman Rho correlation analysis.

as described in Materials and Methods. No difference was noted in the amount of T_{reg} ($CD4^{+}CD25^{+}$ $FoxP3^{+}$) between RA and control patients (Table 3).

Discussion

It has previously been reported that negative selection of T cells in the thymus is controlled by TRAIL [25]. For example, mice deficient in TRAIL had a severe defect in thymic deletion of T cells and were hypersensitive to collagen-induced arthritis [31]. Evidence for functional consequences of TRAIL over-expression in autoimmune diseases including rheumatoid arthritis has emerged from gene therapy studies [32]. Although TRAIL is not cytotoxic to normal cells [33], hyperplastic human RA synovial fibroblasts in culture and RA-activated rabbit synovial tissue *in vivo* were sensitive to adenoviral delivery of human TRAIL [34]. The effect of TRAIL expression on blood lymphocytes was reported to be different (killing versus suppression) than that observed with RA synovial cells. These studies suggested that TRAIL can inhibit the activation and proliferation of lymphocytes *in vivo*, but does not necessarily delete them from the system [35].

The TRAIL/TRAIL receptor system has recently been implicated as a disease activation marker in cancer. High DcR2 expression levels in patients with prostate cancer (PCa) indicate a poorer prognosis, with this marker strongly correlated with high Gleason Scores, Prostate Specific Antigen (PSA) recurrence and decreased survival in PCa patients [36]. In patients with invasive ductal carcinoma, however, high levels of DR4 expression are positively correlated with tumor grade and with poor prognosis [37]. Two recent studies have linked high levels of endogenous TRAIL expression to increased cell death in human pancreatic tissue, supporting the idea that TRAIL might be implicated in the development of autoimmune diseases such as Type 1 Diabetes [38,39]. Despite this information, there is no information concerning the profile of the TRAIL/TRAIL receptor system in the development of RA.

We analyzed peripheral blood lymphocytes of 20 RA patients and 12 control individuals by flow cytometry to detect their TRAIL/TRAIL receptor profile. T cell lymphocytes of healthy control individuals expressed low levels of TRAIL and TRAIL receptors on the cell surface. While this is in accordance with a study conducted by Lu et al. [40], Hasegawa et al. have shown that only DcR2 expression but no other TRAIL receptor expression was detectable on CD8+ T cells [41]. Differences between these studies including ours could be attributed to differences in monoclonal antibodies used. Nevertheless, both $CD4^{+}$ and $CD8^{+}$ T lymphocytes of RA patients displayed higher levels of TRAIL and its receptors on the cell surface compared to healthy control

Table 3 T cell activation marker profile and T_{reg} status in RA versus healthy control patients

Cell Subsets	RA (% cell ± SD)	Control (% cell ± SD)	p value
$CD4^{+}$ $CD25^{+}$	4,9 ± 2,9	3,1 ± 2,0	0,001
$CD4^{+}$ $CD25^{+}$ $FoxP3^{+}$	0,8 ± 1,9	0,6 ± 0,4	0,259

individuals. Since PCa patients can be separated from patients with benign prostate hyperplasia by the amount of TRAIL/TRAIL receptor present in prostate tissue [42] our observations suggest that it might be useful to monitor TRAIL/TRAIL receptor expression in peripheral blood lymphocytes in RA patients at diagnosis and during the course of their disease. The most dramatic change in the amount of TRAIL/TRAIL receptor present on peripheral blood lymphocytes of RA patients was the increased DcR1 expression seen in both $CD4^+$ and $CD8^+$ T cells. Recently, elevated expression of DcR1 was detected in antigen specific T cells of patients with multiple sclerosis (MS) [22]. These antigen specific T cell clones were also resistant to TRAIL mediated apoptosis. One interpretation of these data is that antigen specific T cell clones increase their survival following DcR1 upregulation.

Based on our flow cytometry analysis, RA patients displayed higher levels of activated T cells compared to healthy controls. This finding is in accordance with previous studies demonstrating the presence of higher levels of $CD4^+CD25^+$ peripheral blood lymphocytes in RA patients compared to healthy individuals [43,44]. However we did not observe any change in the level of $CD4^+CD25^+$ $FoxP3^+$ T cells between RA and control groups. In accordance with previous studies, no relationship was found between disease activity and $CD4^+CD25^+$ or $CD4^+CD25^+$ $FoxP3^+$ T cells in RA patients [45]. Whether any subtle increase in TRAIL or TRAIL receptor expression observed on CD4+ T cells in RA patients is simply a consequence of T cell activation remains to be clarified.

Conclusions

Clinical evaluation of RA patients involves RA activity scoring (DAS28). Intriguingly, only the $CD8^+$ T cell associated DR4, DcR1 and DcR2 expression levels correlated with DAS28 scores in patients with RA, implying that altered TRAIL receptor profiles on $CD8^+$ T cell subsets rather than on $CD4^+$ T cells is more important in terms of disease severity.

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Authors' contributions
AB, CA and BY carried out all the assays, ET, NB and VY provided clinical data, RMG and CAA were responsible with flow cytometry procedures, HB acted as scientific consultant, SS coordinated and supervised the study. All the authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Decoy Receptor-2 Small Interfering RNA (siRNA) Strategy Employing Three Different siRNA Constructs in Combination Defeats Adenovirus-Transferred Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Resistance in Lung Cancer Cells

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ABSTRACT

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) selectively induces apoptosis in cancer cells but not in normal cells. However, studies have indicated that more than half of human tumors exhibit TRAIL resistance. Although the mechanism of TRAIL resistance is not understood, it represents a barrier to any TRAIL-mediated gene therapy approach. In addition, no correlation between TRAIL receptor (TRAIL-R) expression profile and TRAIL resistance has been demonstrated in cancer cells. In this study, three different lung cancer cell lines and three different primary cell cultures established from patients with lung cancer (two patients with squamous cell lung carcinoma and one with adenocarcinoma) were screened for sensitivity to adenoviral delivery of TRAIL. Whereas TRAIL-resistant primary lung cell cultures and the A549 lung cancer cell line exhibited high levels of surface decoy receptor-2 (DcR2/TRAIL-R4) expression, TRAIL-sensitive lung cancer cell lines (HBE and H411) failed to express it. A DcR2 short interfering RNA (siRNA) approach involving three different siRNA constructs in combination downregulated DcR2/TRAIL-R4 expression and sensitized lung cancer cells to TRAIL-induced apoptosis. Immunohistochemical staining of samples from 10 patients with lung carcinoma suggested that high-level DcR2/TRAIL-R4 expression is a common phenotype observed in patients with non-small cell lung carcinoma.

OVERVIEW SUMMARY

Lung cancer causes the highest rate of cancer-related death among both men and women. Chemotherapy and radiotherapy are inadequate in increasing patient survival as they both require p53 for their antitumor activity. Death ligands, such as tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL), induce apoptosis regardless of the p53 status of cancer cells. Despite this, TRAIL resistance observed in many tumors represents a handicap for any gene therapy approach targeting cancer. In this study, the A549 lung cancer cell line and primary cell cultures established from three different patients manifested high levels of surface decoy receptor-2 (DcR2/TRAIL-R4) expression and showed resistance to adenoviral delivery of TRAIL. On the other hand, a small interfering RNA (siRNA) cocktail containing three different oligonucleotides designed against DcR2/TRAIL-R4 resulted in the sensitization of lung cancer cells to adenoviral delivery of TRAIL. This study demonstrates that the modulation of TRAIL receptor profiles of cancer cells represents a new therapeutic approach to sensitize cancer cells to TRAIL.

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INTRODUCTION

Lung cancer is the world's leading cause of cancer deaths and causes more deaths than the next three most common cancers (colon cancer, breast cancer, and prostate cancer) combined (Jemal *et al*., 2002). Approximately 1 million people worldwide die of lung cancer yearly (Carney, 2002). On the basis of morphology, lung cancer is divided into two major histological types: non-small cell lung cancer (NSCLC) and small cell lung carcinoma (SCLC). Eighty percent of lung cancer is NSCLC, and 20% is SCLC (Jemal *et al*., 2002). Despite conventional treatment options such as surgery, chemotherapy, and radiotherapy, the 5-year survival rate among patients with NSCLC is only 14%, and the survival rate drops to 5–10% for patients with SCLC (Johnson *et al*., 1990). Because more than 50% of human tumors manifest p53 loss during tumorigenesis, tumors eventually acquire resistance to both radiotherapy and chemotherapy.

Because death ligands induce apoptosis independent of the p53 status of cancer cells, gene therapy approaches involving the administration of death ligands represent a feasible choice for the treatment of radioresistant and chemoresistant cancer cells (Herr and Debatin, 2001). All death ligands tested, including tumor necrosis factor (TNF) (Sanlioglu *et al*., 2004) and Fas ligand (FasL) (Nagata, 1997), efficiently kill cancer cells. Unfortunately, these agents have systemic toxicity, limiting their potential use for cancer gene therapy. In contrast, a novel death ligand, TNF-related apoptosis-inducing ligand (TRAIL) (Wiley *et al*., 1995), has been reported to selectively kill cancer cells without causing any harm to normal cells (Nagane *et al*., 2001). The observation that at least one of the two death receptors is expressed in the majority of locally nonresectable stage III NSCLCs further encourages the use of TRAIL as a death-inducing ligand for lung cancer (Spierings *et al*., 2003). Thus, TRAIL-mediated gene therapy represents an attractive approach for attacking cancer cells without generating systemic toxicity.

Five different receptors interact with TRAIL: TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/DcR1, TRAIL-R4/DcR2, and osteoprotegerin (Abe *et al*., 2000; Sheikh and Fornace, 2000). TRAIL-R1/DR4 and TRAIL-R2/DR5 are the genuine death receptors inducing apoptosis whereas TRAIL-R3/DcR1 and TRAIL-R4/DcR2 function as decoy receptors and do not transmit death signals (Sheridan *et al*., 1997). Activation of TRAIL-R1/DR4 or TRAIL-R2/DR5 by trimeric TRAIL leads to the recruitment of Fas-associated death domain protein (FADD) to the membrane. FADD then recruits procaspase-8 to form the death-inducing signaling complex (DISC). DISC activates the caspase cascade, pushing cells into apoptosis. Caspase-8 is the first apical caspase activated by TRAIL signaling. Caspase-8 in turn activates caspase-3 directly or causes cytochrome *c* release from mitochondria through Bid cleavage. Cytochrome *c* binds to apoptotic protease-activating factor-1 (APAF1) and activates caspase-9, leading to the activation of effector caspases such as caspase-3. Finally, DNA fragmentation and cell death become inevitable.

The fact that TRAIL specifically kills malignant cells but not normal cells led to the testing of systemic TRAIL administration for cancer therapy. However, a significant fraction of the tumor cells displayed TRAIL resistance and the mechanism of TRAIL resistance is not understood (Griffith *et al*., 1998; Sanlioglu *et al*., 2003). Resistance to TRAIL-induced apoptosis can occur at different levels in the TRAIL signaling cascade. First of all, death receptor malfunction due to genetic mutations can lead to TRAIL resistance (Lee *et al*., 1999; Ozoren *et al*., 2000). In addition, death signals cannot be relayed without the proper function of FADD and caspase-8 in the death-inducing signaling complex (Kuang *et al*., 2000; Cheng *et al*., 2006; O'Flaherty *et al*., 2006). Last, the induction of Bcl-2, Bcl-X_L, and inhibitors of apoptosis (IAPs) as well as loss of Bax and Bak function may result in TRAIL resistance (Kandasamy *et al*., 2003; Zender *et al*., 2005; Hamai *et al*., 2006; Petrella *et al*., 2006). Thus, understanding the molecular mechanism of TRAIL resistance in cancer cells is essential to resolve some obstacles associated with the clinical application of TRAIL as a therapeutic agent. So far, no direct connection between the expression profile of TRAIL receptors and TRAIL resistance has been established in cancer cells. In this study, three independent lung cancer cell lines (A549, H411, and HBE) and primary lung cancer cell cultures established from three different patients were tested for sensitivity to TRAIL-induced apoptosis, using an adenoviral vector system (Ad5hTRAIL). Furthermore, TRAIL receptor compositions of lung cancer cells were revealed by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) and flow cytometric analysis. Three different small interfering RNA (siRNA) constructs were generated to knock out the relevant TRAIL decoy receptor expression to reveal its connection to TRAIL resistance in lung cancer cells. Last, an immunohistochemical analysis was performed to determine TRAIL receptor expression profiles of 10 patients with lung cancer.

MATERIALS AND METHODS

Amplification of first-generation recombinant adenoviral vectors

Amplifications of first-generation recombinant adenoviral vectors such as Ad5hTRAIL (Griffith *et al*., 2000), AdEGFP (Sanlioglu *et al*., 2004), and AdCMVLacZ (Sanlioglu and Engelhardt, 1999) were performed as described previously (Engelhardt *et al*., 1993). After CsCl banding and vector purification, adenoviral vectors were kept at -80°C in 10 m*M* Tris containing 20% glycerol. The Ad5hTRAIL construct was used to overexpress hTRAIL in lung cancer cells. Adenoviral vectors expressing the β-galactosidase gene (AdCMVLacZ) were used as a negative control. A_{260} measurements indicated that the particle titers of adenoviral stocks were in the range of 10^{13} DNA particles/ml. Functional titers were measured by plaque titering on 293 cells and by expression assays for encoded proteins. Typically, the particle:plaque-forming unit ratio was equal to 50. All three cell lines used in this work are non-small cell lung carcinoma cell lines of human origin. Specifically, A549 and H411 are adenocarcinoma cell lines. HBE (also known as 16HBE) is a simian virus 40 (SV40) large-T antigen-transformed epithelial cell line derived from human bronchial epithelium.

Efficacy of first-generation adenoviral vector transduction of lung cancer cells

Briefly, lung cancer cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), sodium bicarbonate (2.2 g/liter), 1 m*M* L-glutamine, and 1% penicillin–streptomycin–amphotericin mixture (PSA), using Thermo Scientific Steri-Cult incubators (Thermo Fisher Scientific, Waltham, MA). Adenoviral vectors expressing the enhanced green fluorescent protein (EGFP) reporter gene (AdEGFP) were transferred into lung cancer cells at increasing multiplicities of infection (MOIs) and cells were kept at 37°C in RPMI 1640 without FBS. An equal volume of RPMI 1640 supplemented with 20% FBS was added to increase the serum concentration in the medium to 10% 2 hr after infection. The percentage of $EGFP^+$ cells was determined by fluorescence microscopy and subsequently by flow cytometry 48 hr after infection. Cell viability was assessed by the propidium iodide exclusion technique.

Live/dead cell discrimination

Discrimination of live cells from dead cells was performed with a LIVE/DEAD viability/cytotoxicity kit from Invitrogen Molecular Probes (Eugene, OR). This assay is based on the use of calcein AM and ethidium homodimer-1 (EthD-1). Calcein AM is a fluorogenic substrate for intracellular calcein esterase. It is modified to a green fluorescent compound (calcein) by active esterase in live cells with intact membranes, and thus serves as a marker for viable cells. Unharmed cell membranes do not allow EthD-1, a red fluorescent nucleic acid stain, to enter inside the cell. However, cells with damaged membrane take up the dye and stain positive.

Quantitative real-time RT-PCR assays to detect human TRAIL receptor transcripts

Gene quantification was performed with an Applied Biosystems 7500 real-time PCR system running SDS software (Applied Biosystems, Foster City, CA). Total RNA from lung cancer cells was extracted with TRIzol reagent (Invitrogen Life Technologies, Gaithersburg, MD). TaqMan reverse transcription reagents (cat. no. N8080234; Applied Biosystems) were used to reverse transcribe 2 μg of total RNA. Previously described TRAIL death receptor (Karacay *et al.*, 2004) and TRAIL decoy receptor (Sanlioglu *et al.*, 2005, 2006) primer and probe sequences were used. Both the ribosomal RNA (rRNA) primers and probes were obtained from Applied Biosystems (cat. no. 4308329; Applied Biosystems) and served as an internal control in the same reaction. The $\Delta\Delta C_t$ method as described by Applied Biosystems was used to determine the relative quantities of TRAIL receptors for each sample. The TaqMan PCR was carried out as described by the manufacturer (cat. no. N8080228; Applied Biosystems). For the reverse transcription step, a 50-μl reaction mixture was prepared in reverse transcription buffer with 2.25 m*M* $MgCl_2$, dNTPs (50 μM each), 2.5 μM random hexamer, RNase inhibitor (0.6 U/μl), and reverse transcriptase (1.25 U/μl) diluted in RNase-free distilled water. The thermal cycling conditions were 10 min at 25°C, followed by 60 min at 48°C. TaqMan PCR was performed with TaqMan universal master mix with 50 pmol of primer and probe mixture and 250 ng of cDNA. The thermal cycling conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Each reaction yielded a specific threshold cycle (C_t). ΔC_t values were then calculated for each receptor by taking the difference between the C_t values of the TRAIL receptors and that of rRNA internal controls. Apart from this, a standard curve was established on the basis of serial dilutions of rRNA. The comparative $\Delta\Delta C_t$ calculation was then performed by finding the difference between the ΔC_t value of each receptor and that of 25 pg of rRNA. Final relative expression levels were estimated by the formula $2^{-\Delta\Delta C_t}$. By doing this, relative expression levels of TRAIL receptors are normalized to that of 25 pg of ribosomal RNA.

Flow cytometric detection of TRAIL receptor protein expression on the cell surface

TRAIL receptor protein expression on the cell surface was detected with an anti-TRAIL receptor flow cytometry set (cat. no. ALX-850-273-KI01; Alexis Biochemicals, Lausen, Switzerland). This kit contains 100 μg of monoclonal antibody (mAb) to TRAIL-R1 (clone HS101; cat. no. ALX-804-297A), TRAIL-R2 (clone HS201; cat. no. ALX-804-298A), TRAIL-R3 (clone HS301; cat. no. ALX-804-344A), and TRAIL-R4 (clone HS402; cat. no. ALX-804-299A). Primary antibodies were used at a concentration of 5 μg/ml. Biotinylated goat anti-mouse IgG1 (cat. no. ALX-211-202; Alexis Biochemicals) served as a secondary antibody. Streptavidin–phycoerythrin (PE) (cat. no. ANC-253-050; Ancell, Bayport, MN) was added before flow cytometry was performed. A flow cytometry instrument (EPICS ALTRA with HyPerSort cell sorting; Beckman Coulter, Fullerton, CA), located at the Human Gene Therapy Unit of Akdeniz University Hospitals and Clinics, was used to carry out flow cytometric analysis. Purified mouse IgG1 (MOPC 31C; cat. no. ANC-278-010; Ancell) was used as an isotype control. Monoclonal antibody to TRAIL (human) (cat. no. ALX-804-296-C100; Alexis Biochemicals) was applied, followed by polyclonal antibody to mouse IgG1 (R-PE) (cat. no. ALX-211-201-C050; Alexis Biochemicals), to reveal TRAIL expression on the cell surface.

TRAIL-R4 gene silencing by siRNA approach

Decoy receptor-2 (DcR2) siRNA experiments were conducted with DcR2 siRNA (cat. no. sc-35185; Santa Cruz Biotechnology, Santa Cruz, CA), siRNA transfection medium (cat. no. sc-36868), and siRNA transfection reagent (cat. no. sc-29528) in lung cancer cells as described by the manufacturer. Product sc-35185 is a pool of three siRNA sequences (to increase the silencing effect) designed for TRAIL-R4 mRNA silencing (GenBank accession number NM_003840). Lung cancer cells were plated in 24-well plates at a density of 2×10^5 cells per well after being resuspended in fresh RPMI 1640 containing 10% fetal bovine serum without antibiotics. Cells were then grown overnight to 40–50% confluency. The next day, siRNA–siRNA transfection reagent complex was prepared as a mixture of transfection solutions A and B (solution A: 1.8 μl of 10 μM siRNA mixed with 30 μl of siRNA transfection medium; solution B: 1.8 μl of siRNA transfection reagent

mixed with 7.25 μl of siRNA transfection medium), and incubated at room temperature for 20 min. The optimal dose of DcR2 siRNA needed to block TRAIL-R4 expression without serious cytotoxic effects was determined on the basis of a concentration gradient. Medium was removed from cells in the 24-well plate, and 300 μl of fresh growth medium (10% serum, without antibiotics) was added to each well. The transfection reagent complex was then added to the cells dropwise while gently rocking the plate. After transfection, cells were incubated for 30 hr at 37°C. After 30 hr, cells were infected with increasing MOIs of either Ad5hTRAIL viral vector or Ad-CMVLacZ at a constant MOI of 10,000 DNA particles/cell. Uninfected plus untransfected, uninfected plus transfected, and infected plus untransfected cells were used as controls. Transfection efficiency was originally determined with fluorescein-conjugated control siRNA-A (cat. no. sc-36869) under a fluorescence microscope. More than 95% of the cells were transfected by this procedure. Control siRNA-A (sc-37007) containing scrambled sequences that did not lead to the specific degradation of any cellular RNA was used as a negative control in siRNA experiments.

In vitro *siRNA synthesis*

Desalted DNA oligonucleotides encoding top- and bottom-strand target sequences (Table 1) were ordered from Integrated DNA Technologies (Coralville, IA). T7 promoter sequence (Table 1) was added at the 3′ ends of each primer for *in vitro* transcription. *In vitro* synthesis of siRNA was carried out with an AmpliScribe T7 high-yield transcription kit (Epicentre Biotechnologies, Madison, WI). Briefly, 1 μg of target oligonucleotide was mixed with 1 μg of T7 promoter oligonucleotide by heating at 95°C for 5 min, after which the heating block was switched off and allowed to cool slowly to 70°C. The tube was moved to a 37°C water bath for an additional 15 min to obtain annealed, double-stranded DNA (dsDNA). *In vitro* synthesis of siRNA was performed according to the manufacturer's protocol. The transcription mix included 1× T7 AmpliScribe buffer, 7.5 m*M* rNTPs, 10 m*M* dithiothreitol (DTT), T7 RNA polymerase, and 1 μg of dsDNA as a template. After incubation at 37°C for 2 hr, 1 U of RNase-free DNase I was added at 37°C for 15 min. To obtain small interfering double-stranded RNA, top- and bottom-strand RNAs generated in separate reactions were annealed by the following procedure. The two transcription reactions were mixed and heated at 95°C for 5 min. The heating block was switched off and allowed to cool slowly to 70°C. The reaction tube was then transferred to 37°C for an additional 15 min. siRNA was precipitated with 3 volumes of ethanol after adding 100 μl of nuclease-free water with 1 m*M* EDTA and 0.125 *M* LiCl.

Table 1. Primer Sequences for the Generation of DcR2 siRNA Oligonucleotides[a]

TRAIL-R4 siRNA-1	
Top:	GGATGGTCAAGGTCAGTAA
Bottom:	TTACTGACCTTGACCATCC
TRAIL-R4 siRNA-2	
Top:	CCCTATCACTACCTTATCA
Bottom:	TGATAAGGTAGTGATAGGG
TRAIL-R4 siRNA-3	
Top:	GCTTGGGAATGGTGTGAAA
Bottom:	TTTCACACCATTCCCAAGC

Abbreviations: DcR2, decoy receptor-2; siRNA, small interfering RNA; TRAIL-R4, tumor necrosis factor-related apoptosis-inducing ligand receptor-4.

[a]The primer 5′-TAATACGACTCACTATAG-3′ is annealed to all oligonucleotides to synthesize siRNAs.

TRAIL-R4 cDNA transfection and RNA interference assays

Monkey kidney fibroblast Cos-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and penicillin–streptomycin mix, and human lung A549 cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal calf serum and penicillin–streptomycin mix, at 37°C in a 5% CO_2 atmosphere. Cells were transfected with Lipofectamine Plus reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. Cos-7 cells (2×10^5) were transfected with plasmid vector carrying human TRAIL-R4 cDNA (a gift of T.S. Griffith, University of Iowa, Iowa City, IA) alone, or in combination with *in vitro*-transcribed siRNAs for TRAIL-R4. Cells were transfected with 0.3 μg of human TRAIL-R4 cDNA expression vector and 1.5 μg of siRNA (1:5 ratio). The control group was transfected with 0.3 μg of TRAIL-R4 cDNA expression vector. To measure the silencing of endogenous TRAIL-R4 gene expression, the A549 human lung cancer cell line was transfected as described above with *in vitro*-transcribed siRNAs for TRAIL-R4, separately or in combination. Twenty-four hours after transfection, RNA was isolated with TRIzol reagent for subsequent real-time RT-PCR assay as described above.

Establishment of primary cell cultures from patients with lung carcinoma

Immediately after the surgical removal of lung carcinoma tissue from patients, the tissue was immersed in serum-free RPMI 1640 medium containing antibiotics, and kept cold while being transported to the laboratory. A microdissection technique was used to directly obtain samples from each of the tumors. Lung cancer tissue was then minced into small pieces with forceps and scissors in a sterile 10-cm Petri dish. Minced tissue samples were treated with collagenase type II (300 units/ml [cat. no. G6885]; Sigma, St. Louis, MO) in RPMI 1640 at 37°C for 2.5 hr with gentle stirring. After incubation, the cells were collected by gentle pipetting into a centrifuge tube. After centrifugation, the cells were washed twice with culture medium without FBS. After washing, the cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, insulin (15 μg/ml [cat. no. I1882]; Sigma), human epidermal growth factor (20 ng/ml [cat. no. E9644]; Sigma), and fibronectin (100 ng/ml [cat. no. F4759]; Sigma) at 37°C in a humidified 5% CO_2 incubator. In addition, two methods were used to eliminate possible fibroblast contamination in culture. The first was a partial trypsinization procedure in which a low concentration of trypsin solution (0.05%) was used. This protocol is based on the principle that fibroblasts are easier to detach than epithelial cells. The second approach involved the performance of a differential detachment technique. This protocol, based on the princi-

ple that fibroblasts attach to a dish surface faster than do epithelial cells, involves plating cells for a short period of time (2–6 hr), recovering unattached cells, and finally plating them into a new dish. The incubation period lasted several weeks before conducting our molecular assays.

Immunohistochemistry of TRAIL and its cognate receptors in patients with lung cancer

All primary antibodies were obtained from Alexis Biochemicals. The following primary antibodies (diluted 1:300) were deployed for the immunohistochemical analysis of lung specimens: Monoclonal antibody to TRAIL-R1 (human [HS101]; cat. no. ALX-804-297A-C100), polyclonal antibody to TRAIL-R2 (cat. no. ALX-210-743-C200), polyclonal antibody to TRAIL-R3 (human; cat. no. ALX-210-744-C200), and monoclonal antibody to TRAIL-R4 (human [HS402]; cat. no. ALX-804-299A-C100). The specificity of these primary antibodies was previously confirmed by Alexis Biochemicals. Specimens were immunostained in the absence of primary antibodies and these were used as negative controls. No immunostaining was done when primary antibodies were not used.

Immunohistochemical scoring of TRAIL and TRAIL receptors for patients with lung cancer

Slides of the specimens were analyzed by an independent pathologist (G.O.) who had no prior knowledge of the clinical data. Immunostaining scores were given on the basis of both the intensity and marker distribution (percentage of positively stained epithelial cells) in lung carcinoma as described previously (Sanlioglu *et al*., 2007). Intensity was scored as follows: 0, negative; 1, weak; 2, moderate; and 3, strong staining. Marker distribution was scored as follows: 0, less than 10%; 1, between 10 and 40%; 2, between 40 and 70%; and 3, more than 70% of the epithelial cells stained on the specimen. The final immunostaining score was obtained by adding the scores for both intensity and marker distribution for a given patient. SPSS 13.0 software for Windows (SPSS, Chicago, IL) was used for statistical analysis. Normality of the patient groups (adenocarcinoma [AC] and squamous cell lung carcinoma [SCC]) was tested by the Shapiro–Wilk method. None of the groups displayed a Gaussian distribution. For this reason, the significance of differences among the groups was determined by Friedman test. Later, the Wilcoxon signed ranks test with Bonferroni's correction was applied to compare paired groups of two.

RESULTS

First-generation adenoviral vectors efficiently transduce lung cancer cells

Before performing TRAIL transfer into lung cancer cells, the efficacy of the first-generation recombinant adenoviral vector transduction of lung cancer cells was revealed with an adenoviral vector encoding enhanced green fluorescent protein (AdEGFP). The main rationale for performing these assays was to determine the optimal dose of adenovirus to conduct gene delivery. The efficacy of viral infection was monitored by fluorescence microscopy, and the transduction results were quantitatively analyzed by flow cytometry 48 hr after infection. Almost 100% of the cells were efficiently transduced with AdEGFP at an MOI of 5000 DNA particles/cell 48 hr after infection (data not shown).

A549 lung carcinoma cell line is resistant to adenoviral delivery of hTRAIL

Despite the fact that the death ligand TRAIL exerts anticancer properties, the observed TRAIL resistance of cancer cells has critically challenged the use of TRAIL as a gene therapy agent. To find out whether A549 lung cancer cells are resistant to TRAIL, an adenoviral vector encoding hTRAIL (Ad5hTRAIL) or β-galactosidase (AdCMVLacZ) was transferred at increasing doses into A549 lung cancer cells. Infection with neither Ad5hTRAIL (MOI of up to 10,000 DNA particles/cell) nor AdCMVLacZ (control, data not shown) vector reduced the amount of viable cells 48 hr after infection, as depicted in Fig. 1A. To rule out the possibility that the failure to observe any cytotoxic effect was due to the lack of TRAIL expression from the vector, a flow cytometric analysis was performed with A549 cells infected with Ad5hTRAIL (Fig. 1B). This assay demonstrated that significant levels of TRAIL overexpression were achieved through infection of A549 cells with Ad5hTRAIL.

TRAIL receptor expression profile of A549 lung cancer cells

Despite extensive efforts to determine the mechanism of TRAIL resistance in cancer cells, no connection between the expression pattern of TRAIL receptors and TRAIL sensitivity has been demonstrated (Griffith *et al*., 1998). Quantitative real-time RT-PCR assays were carried out with primer–probe sets specifically designed to detect each of the TRAIL receptors in A549 lung cancer cells (Fig. 2A). Although all TRAIL receptors were present in A549 lung cancer cells, TRAIL-R2 death receptor gene expression was the highest among the four. TRAIL-R1 and TRAIL-R4 receptor gene expression was evident at similar levels, and TRAIL-R3 decoy receptor gene expression was relatively low compared with the other three receptors. Conventional flow cytometric analysis was conducted with antibodies specific to each of the TRAIL receptors, because mRNA expression inside the cell may not necessarily correlate with protein expression on the cell surface. Results of this assay suggested that all four TRAIL receptors were expressed on the surface of A549 lung cancer cells (Fig. 2B). Substantial levels of TRAIL-R4 decoy receptor protein expression were evident on the surface of A549 lung cancer cells. TRAIL-R3 decoy receptor protein expression was lowest among the four but was, nonetheless, still detectable on the cell surface.

DcR2 siRNA approach sensitizes A549 lung cancer cells to TRAIL

A DcR2 siRNA approach was used to attenuate or block TRAIL-R4 decoy receptor expression in A549 lung cancer cells. Downregulation of TRAIL-R4 protein expression following DcR2 siRNA administration was confirmed by flow cytometry (Fig. 3A). Expression of none of the TRAIL receptors was affected when control siRNA-A was used as a negative

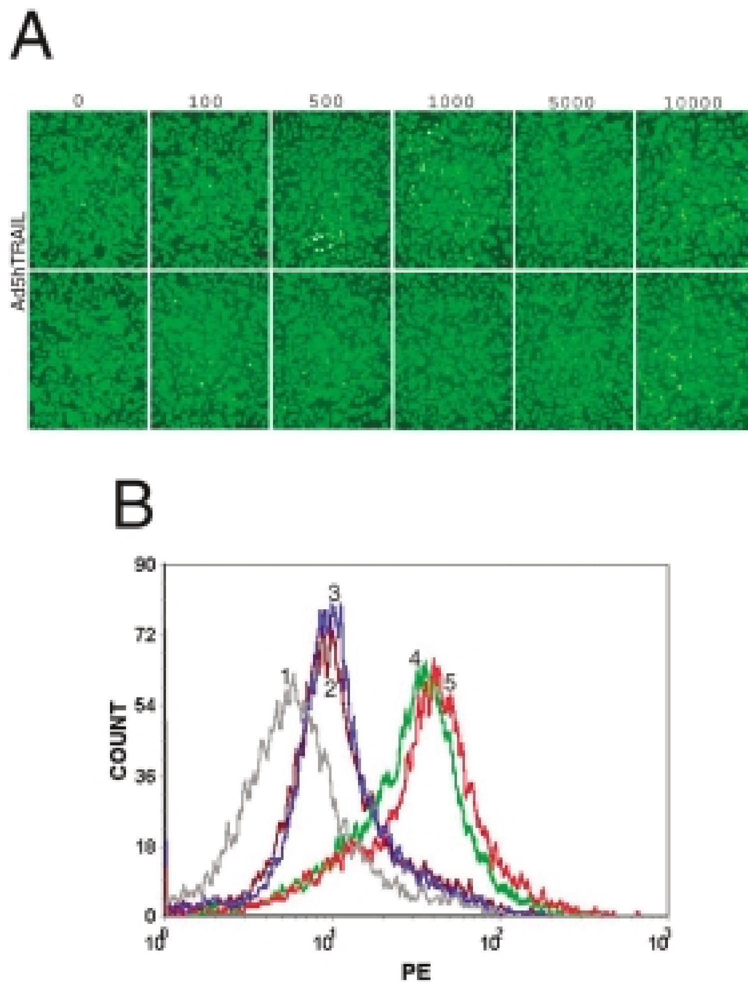

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FIG. 1. A549 lung cancer cells exhibit TRAIL resistance. (**A**) Ad5hTRAIL vector was transferred into A549 lung cancer cells at increasing doses as described in Materials and Methods. An Invitrogen Molecular Probe LIVE/DEAD viability/toxicity kit was used to detect viable cells 48 hr after infection. The viral doses applied are presented as MOI values (DNA particles per cell) above each panel. Duplicate samples are given for each infection. (**B**) Flow cytometric analysis of hTRAIL expression in A549 cells infected with Ad5hTRAIL. Conditions for infection were as follows: 1, unstained; 2, uninfected (secondary antibody alone); 3, infected with AdLacZ; 4, infected with Ad5hTRAIL at an MOI of 5000 DNA particles/cell; 5, infected with Ad5hTRAIL at an MOI of 10,000 DNA particles/cell.

control for the transfection instead of DcR2 siRNA. Then, either Ad5hTRAIL or AdCMVLacZ (negative control) infection was performed at increasing doses after DcR2 siRNA administration in A549 lung cancer cells. Proportions of live and dead cells were determined 48 hr after infection. Although A549 lung cancer cells were previously shown to be resistant to TRAIL, downregulation of TRAIL-R4 decoy receptor expression resulted in the sensitization of A549 lung cancer cells to Ad5hTRAIL (Fig. 3B). No such effect was observed when A549 lung cancer cells were infected with AdCMVLacZ virus (Fig. 3B). TRAIL-R4 decoy receptor expression was reduced by 85 ± 7% after DcR2 siRNA administration as demonstrated by flow cytometry. In addition, quantitative real-time RT-PCR analysis was performed in order to determine the level of TRAIL-R4 RNA after DcR2 siRNA administration. This assay indicated a 75 ± 5% decrease in TRAIL-R4 RNA levels.

Because the DcR2 siRNA kit (cat. no. sc-35185) from Santa Cruz Biotechnology consists of three siRNA constructs in combination developed against TRAIL-R4 mRNA, custom synthesis of individual siRNA constructs was performed by a completely different approach as described in Materials and Methods in order to check the efficacy of individual siRNAs for their inhibitory effect on TRAIL-R4 expression (Table 1). As a first step, monkey kidney fibroblast Cos-7 cells were transfected with TRAIL-R4-encoding cDNA alone or in combination with individual *in vitro*-transcribed siRNAs. A nonhuman cell line was chosen for the proof-of-principle experiments because the lack of TRAIL-R4 expression on these cells provided a proper control. As seen in Fig. 4A, all three double-stranded siRNAs effectively downregulated TRAIL-R4 mRNA expression on Cos-7 cells. After confirming that *in vitro*-transcribed siRNAs effectively interfered with the expression of ectopically expressed TRAIL-R4 the silencing effect of the same siRNAs was examined in A549 cells. As demonstrated in Fig. 4B, siRNA-3 and the combination of three siRNAs were effective in reducing TRAIL-R4 expression to an extent greater than 80% (Fig. 4B). The level of silencing was similar to the result obtained with the DcR2 siRNA kit (cat. no. sc-35185; Santa Cruz Biotechnology). Infection with Ad5hTRAIL at an MOI of 5000 DNA particles/cell resulted in a 65 ± 6% decrease in cell viability after transfection with the siRNA cocktail containing three oligonucleotides in combination. However, some differences were also observed between the two sets of experiments. This difference, between Cos-7 and A549 cells, can be attributed to the experimental setup consisting of an overexpression scenario (Cos-7 cells) versus endogenous levels of TRAIL-R4 mRNA (A549

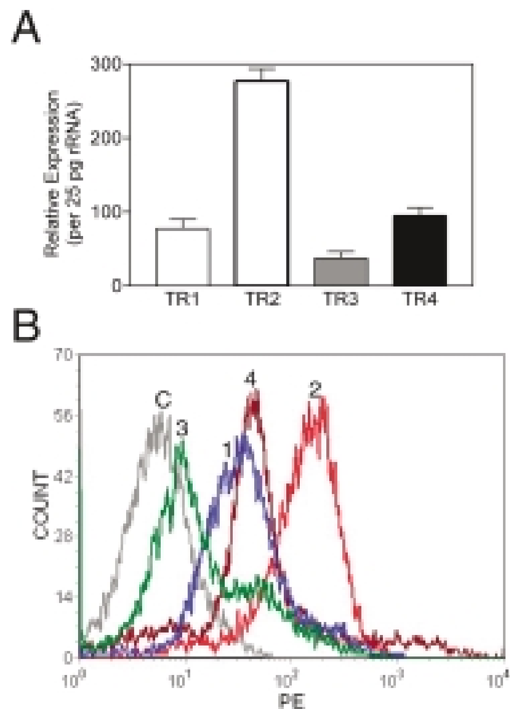

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FIG. 2. TRAIL receptor profile of A549 lung cancer cells. (**A**) A real-time RT-PCR assay was performed to quantify the amounts and types of TRAIL receptor expressed on A549 lung cancer cells. Ribosomal RNA primers and probes were included in each TaqMan reaction as an internal control. Relative expression per 25 pg of rRNA is provided on the *y* axis. (**B**) Surface TRAIL receptor profile of A549 lung cancer cells. Conventional flow cytometric analyses were performed to detect the surface expression profile of TRAIL receptors as explained in Materials and Methods. Peak C, isotype control staining; peaks 1–4, TRAIL-R1 through TRAIL-R4, respectively. A total of 10^4 A549 lung cancer cells was gated for each histogram. This assay was repeated three times, but only one representative assay is shown for clarity.

cells). Nevertheless, in both sets of experiments, generated siRNAs substantially reduced the expression of TRAIL-R4.

TRAIL-sensitive H411 and HBE lung carcinoma cell lines express all TRAIL receptors except TRAIL-R4

To strengthen the correlation between the decoy receptor gene expression and TRAIL resistance observed in A549 cells, two other non-small cell lung carcinoma cell lines were screened. Interestingly, neither H411 nor HBE lung carcinoma cells exhibited detectable levels of TRAIL-R4 decoy receptor expression on the cell surface as demonstrated by flow cytometric analysis (Fig. 5). Ad5hTRAIL infection alone at an MOI of 5000 DNA particles/cell resulted in 80% reduction in the viability of HBE cells and 73% reduction in the viability of H411 cells. Obviously, surface TRAIL-R3 decoy receptor expressed on both of these cell lines was not able to protect the cells from TRAIL-induced cytotoxicity. These results may suggest that in the absence of TRAIL-R4 expression on the cell surface, TRAIL death receptors are the main modulators of TRAIL sensitivity in lung cancer cells.

TRAIL-resistant primary lung carcinoma cells display high levels of TRAIL-R4 decoy receptor expression on the cell surface

To solidify the connection between the expression pattern of TRAIL receptors and TRAIL sensitivity, primary cell cultures were established from three patients with non-small cell lung carcinoma (NSCLC). Two of these patients had squamous cell lung carcinoma (SCC) and one had adenocarcinoma (AC). SCC cases were diagnosed on the basis of immunohistochemical staining of paraffin-embedded sections (Fig. 6A). A real-time RT-PCR assay conducted with a primary cell culture established from the first patient with SCC indicated that although all four TRAIL receptors were expressed in this patient, TRAIL-R4 decoy receptor expression was the highest, based on mRNA levels (Fig. 6B). In addition, only TRAIL-R4 decoy receptor expression was evident on the surface of primary lung cancer cells

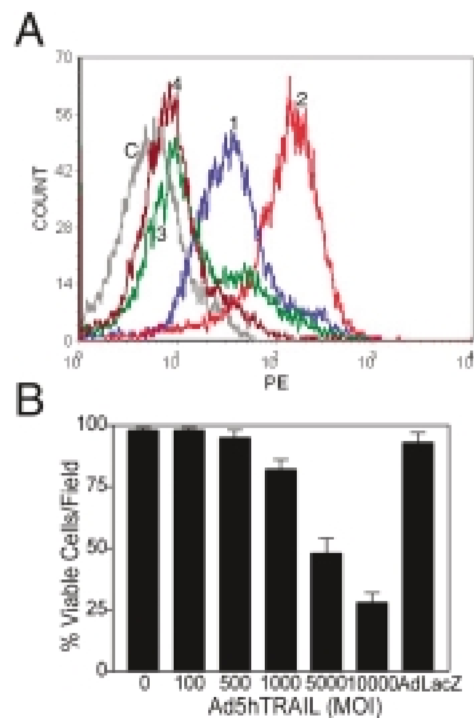

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FIG. 3. Downregulation of TRAIL-R4 expression sensitizes A549 lung cancer cells to TRAIL. (**A**) DcR2 siRNA approach targeting TRAIL-R4 expression in A549 lung cancer cells. Other TRAIL receptor expressions are shown as controls. C represents isotype control staining. Each histogram represents 10^4 gated A549 lung cancer cells. The number above each peak corresponds to a specific TRAIL receptor (1 = TRAIL-R1, etc.). One of three independent flow assays is given. Downregulation of TRAIL-R4 decoy receptor gene expression knocks out TRAIL resistance in A549 lung cancer cells (**B**). The DcR2 siRNA approach was conducted as described in Materials and Methods. AdLacZ in (**B**) refers to AdCMVLacZ virus infected at an MOI of 10,000 DNA particles/cell into A549 cells after DcR2 siRNA transfection in place of Ad5hTRAIL virus. Data represent means and SEM of three independent assays ($n = 6$).

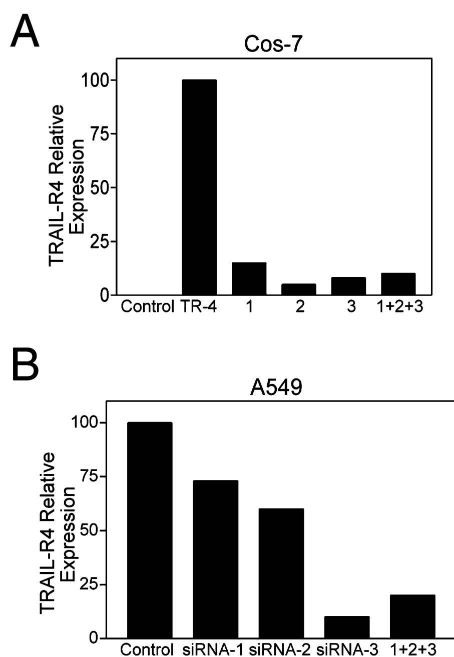

FIG. 4. *In vitro*-synthesized siRNA constructs downregulate TRAIL-R4 expression in Cos-7 cells (**A**) and A549 cells (**B**). siRNA constructs (siRNA-1 to siRNA-3), displayed on the *x* axis, were synthesized as described in Materials and Methods. Except for the control samples, Cos-7 cells were transfected with TRAIL-R4 cDNA before the siRNA assay, as shown in (**A**). TRAIL-R4 expression levels were detected by real-time RT-PCR assay after transfection. Treatment conditions are provided on the *x* axis. A 1.5-μg amount of each siRNA was used in transfection with the exception of combined siRNA administration (column 1+2+3), in which 0.5 μg of each siRNA construct (total, 1.5 μg) was used. Experiments were repeated twice to confirm the observations; data from only one representative assay are provided.

as detected by flow cytometry (Fig. 6C). No TRAIL receptor other than TRAIL-R4 was expressed at detectable levels on the surface (data not shown for clarity). As expected, primary lung carcinoma cells were completely resistant to adenoviral delivery of TRAIL up to an MOI of 10,000 DNA particles/cell (data not shown). Furthermore, the DcR2 siRNA approach downregulated surface TRAIL-R4 expression (peak marked with an asterisk in Fig. 6C) but still failed to sensitize cells to adenoviral delivery of TRAIL. Although the lack of death receptor gene expression can also account for TRAIL resistance in this particular case, expression of high levels of TRAIL-R4 decoy receptor on the cell surface may imply that this phenotype is not restricted only to the A549 lung carcinoma cell line. Real-time RT-PCR analysis of a primary cell culture established from the second patient with SCC indicated, on the basis of mRNA levels, that all four TRAIL receptors were expressed (Fig. 7A). Flow cytometric analysis demonstrated that higher levels of TRAIL-R4 decoy receptor were expressed on the cell surface compared with TRAIL death receptors TRAIL-R1 and TRAIL-R2. But TRAIL-R3 decoy receptor expression was not detectable on the surface. DcR2 siRNA transfection, but not siRNA-A, downregulated (86%) surface TRAIL-R4 expression (Fig. 7B). Later, infection with Ad5hTRAIL, but not AdCMVLacZ, at an MOI of 10,000 DNA particles/cell, resulted in TRAIL sensitization (42 ± 5%) of DcR2 siRNA-transfected primary lung cancer cells (data not shown). The third patient was diagnosed with AC. The primary cell culture established from this patient manifested higher levels of TRAIL-R2 and TRAIL-R4 mRNA expression compared with TRAIL-R1, as demonstrated by real-time RT-PCR assays (Fig. 8A). In this case, TRAIL-R3 decoy receptor mRNA expression was not detectable. Accordingly, only TRAIL-R2 and TRAIL-R4 receptors were evident on the cell surface as shown by flow cytometric analysis (Fig. 8B). Next, surface TRAIL-R4 expression was downregulated by the DcR2 siRNA approach (76%), but not by administration of siRNA-A, as presented in Fig. 8B. This resulted in TRAIL sensitization of lung cancer cells treated with Ad5hTRAIL, but not with AdCMVLacZ, at an MOI of 10,000 DNA particles/cell to an extent greater than 68 ± 6% (data not shown).

NSCLC patients display higher levels of TRAIL-R4 expression compared with other TRAIL receptors

To investigate the *in vivo* relevance of the preceding findings, TRAIL receptor profiles of patients with lung cancer were examined by immunohistochemical analysis of paraffin-embedded sections obtained from patients either with AC or SCC (Fig. 9A). On the basis of blinded scoring as described in

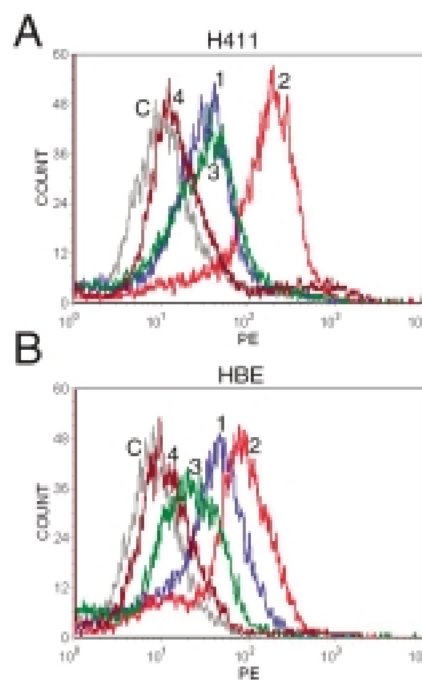

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FIG. 5. TRAIL-sensitive lung cancer cell lines (H411 and HBE) displayed undetectable levels of TRAIL-R4 expression on the cell surface. Flow cytometric analysis and Ad5hTRAIL infections were performed as described in Materials and Methods. A total of 10^4 lung cancer cells was gated for each histogram. Peak C, isotype control staining; peaks 1–4, TRAIL-R1 through TRAIL-R4, respectively. Assays were performed in duplicate to confirm the results.

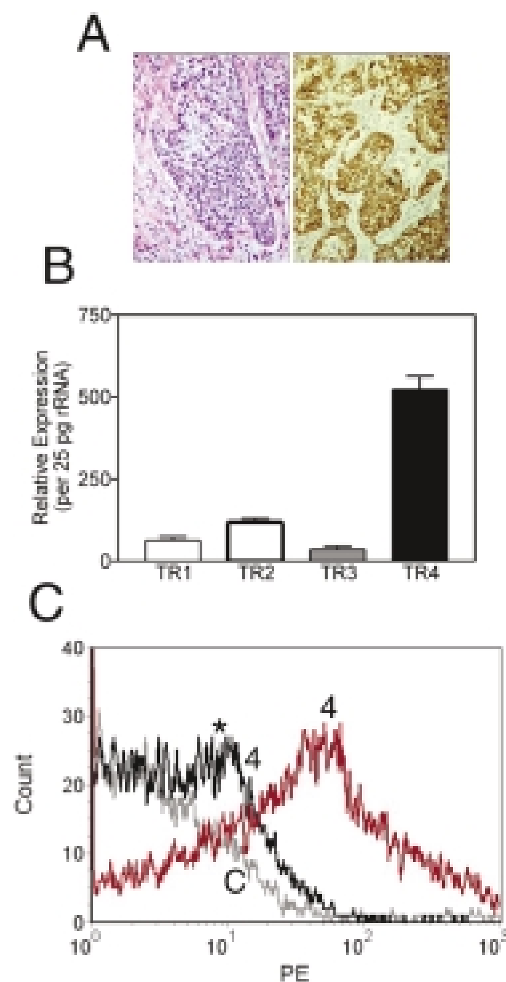

FIG. 6. Decoy receptor TRAIL-R4 is the main receptor expressed in a primary cell culture from a patient with SCC. The primary lung cell culture was prepared as described in Materials and Methods. (**A**) H&E staining (*left panel*) of lung cancer tissue and high molecular weight keratin-positive cells (*right panel*), which indicate squamous cell lung carcinoma. (**B**) Real-time RT-PCR assay displaying intracellular TRAIL receptor composition at the mRNA level. (**C**) Flow cytometric analysis of the same primary lung carcinoma cell culture. A total of 10^4 lung cancer cells was gated for each histogram. The results of one of two independent assays is shown. Peak C, isotype control staining; peak 4, TRAIL-R4; peak 4*, DcR2 siRNA-transfected TRAIL-R4 sample.

Materials and Methods, both SCC and AC patients displayed higher levels of TRAIL-R4 expression compared with other types of TRAIL receptors (Fig. 9B).

DISCUSSION

It has been reported that at least 50% of human tumors exhibit resistance to TRAIL (Griffith and Lynch, 1998). In accordance with this, two different hypotheses are asserted to explain TRAIL resistance. The first hypothesis suggests the presence of decoy receptors that compete for binding to TRAIL (Sheridan *et al*., 1997; Srivastava, 2001). According to this theory, these receptors either dilute out TRAIL ligands (such as TRAIL-R3) or supply antiapoptotic signals (like TRAIL-R4) to cells (Chaudhary *et al*., 1997; Pan *et al*., 1997). However, RNase protection assays did not reveal any connection between TRAIL sensitivity and the expression of TRAIL-R1, TRAIL-R2, and TRAIL-R3 in lung cancer cell lines (Kagawa *et al*., 2001). Although this particular study also demonstrated that A549 lung cancer cells were relatively resistant to a TRAIL-mediated gene therapy approach, TRAIL-R4 expression in the A549 cell line was not examined. The second hypothesis claims the presence of apoptosis-inhibitory molecules that counteract TRAIL-mediated apoptosis (French and Tschopp, 1999a). For example, the level of c-FLIP (cellular FLICE [FADD-like interleukin-1β-converting enzyme]-inhibitory protein) expression has previously been blamed for TRAIL resistance in cancer cells (French and Tschopp, 1999b). However, screening of NSCLC cell lines by Western blotting did not reveal any correlation between the expression pattern of c-FLIP and TRAIL resistance (Frese *et al*., 2002). Contrary to expectation, TRAIL-sensitive cell lines (NCI-H358) expressed higher levels of c-FLIP, whereas low levels of c-FLIP were observed in TRAIL-resistant cell lines (A549, Calu1, and SkLu1). Accordingly, it

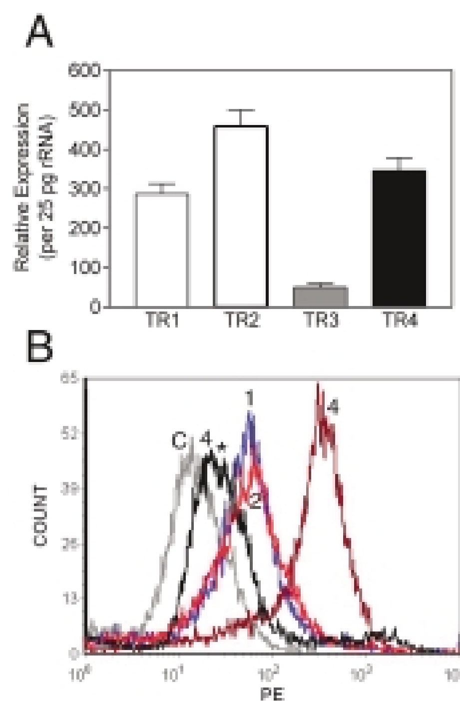

FIG. 7. Quantitative RT-PCR (**A**) and flow cytometric analysis (**B**) of a primary cell culture established from the second patient with SCC. Relative expression per 25 pg of rRNA is given on the *y* axis for the real-time PCR assay. Data represent means and SEM of two independent assays ($n = 6$). Flow cytometric analyses were conducted after transfection either with DcR2 siRNA or siRNA-A. A total of 10^4 lung cancer cells was gated for each histogram. The results of one of two independent assays is shown. Peak C, isotype control staining; peaks 1, 2, and 4, TRAIL-R1, TRAIL-R2, and TRAIL-R4, respectively; peak 4*, DcR2 siRNA-transfected TRAIL-R4 sample.

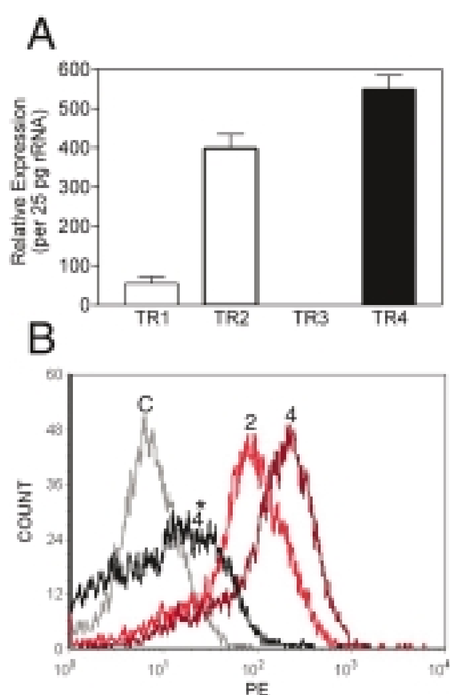

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FIG. 8. TRAIL receptor profile of a primary cell culture established from a patient with AC. (**A**) The level of TRAIL receptor transcripts as assessed by real-time RT-PCR analysis. The *y* axis shows relative expression per 25 pg of rRNA. Data represent means and SEM of two independent assays ($n = 6$). (**B**) Flow cytometric analysis indicating the surface TRAIL receptor expression pattern revealed after transfection either with siRNA-A or DcR2 siRNA. A total of 10^4 lung cancer cells was gated for each histogram. The results of only one of two independent experiments are shown. Peak C, isotype control staining; peaks 2 and 4, TRAIL-R2 and TRAIL-R4, respectively; peak 4*, DcR2 siRNA-transfected TRAIL-R4 sample.

was concluded that mechanisms other than the level of c-FLIP expression might be responsible for TRAIL resistance in NSCLCs.

Although efforts have been made to elucidate the molecular mechanism of TRAIL resistance, no direct correlation between the expression pattern of TRAIL receptors and TRAIL resistance has been reported (Kagawa *et al.*, 2001). To assess the level of TRAIL receptor gene expression in A549 lung cancer cells, quantitative real-time RT-PCR assays were performed. This assay demonstrated that all four TRAIL receptors were expressed in A549 lung cancer cells, and that the expression level of TRAIL-R2 death receptor was the highest among the four. Because mRNA levels inside the cell may not correlate with protein expression on the cell surface (the latter being more relevant for TRAIL sensitivity), we performed flow cytometric analysis. We found that although all TRAIL receptors were expressed on the cell surface, there were substantial levels of TRAIL-R4 decoy receptor protein expression on the surface of A549 lung cancer cells. Interestingly, it has previously been reported that TRAIL-R4 overexpression protected target cells from TRAIL-induced cytotoxicity (Degli-Esposti *et al.*, 1997). This report claimed that transient TRAIL-R4 expression protected cells from apoptosis by acting both as a decoy receptor and as an antiapoptotic signal provider. In addition, adenovirus-mediated *p53* delivery upregulated TRAIL-R4 mRNA expression, delaying TRAIL-, p53-, and TRAIL-R2-dependent colon cancer apoptosis (Meng *et al.*, 2000). To reveal the possible role of TRAIL-R4 expression in the constitution of TRAIL resistance, a DcR2 siRNA approach was applied before the infection of A549 lung cancer cells with Ad5hTRAIL. Intriguingly, downregulation of TRAIL-R4 expression sensitized TRAIL-resistant A549 lung cancer cells to TRAIL. In addition, two other NSCLC cell lines (H411 and HBE), which expressed undetectable levels of TRAIL-4 decoy receptors on the cell surface, were tested for sensitivity to the Ad5hTRAIL vector. Despite the expression of TRAIL-R3 decoy receptor on the surface these two cell lines were relatively sensitive to TRAIL overexpression. To solidify the connection between TRAIL-R4 expression and TRAIL resistance, primary cell cultures were established from three patients with NSCLC. All primary cell cultures expressed high levels of TRAIL-R4 decoy receptor on the cell surface and all were resistant to adenoviral delivery of TRAIL. DcR2 siRNA, but not siRNA-A, transfection sensitized these primary cell cultures to the cytotoxic effects of TRAIL as long as a death receptor was present on the cell surface. All these results may suggest that TRAIL-R4 expression on the cell

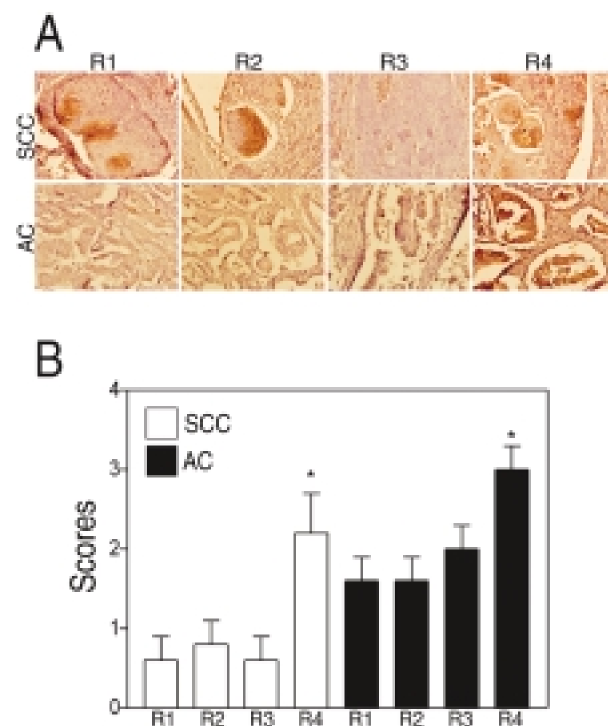

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FIG. 9. Immunohistochemical analysis of TRAIL and TRAIL receptors in patients with lung cancer. Five patients with SCC and five other patients with AC were analyzed in terms of TRAIL and TRAIL receptor gene expression, using immunohistochemistry. Bright-field images are shown in (**A**). Immunohistochemical staining scores are provided in (**B**). Procedures for immunohistochemical staining and scoring and statistical analyses are described in Materials and Methods. $*p < 0.05$, compared with other TRAIL receptors.

surface drastically influences TRAIL sensitivity in cancer cells. Consequently, testing of primary lung cancer cells established from patients as well as immunohistochemical analysis of 10 other patients with NSCLC demonstrated that high-level TRAIL-R4 decoy receptor expression was a common phenotype observed in patients with lung cancer. These results suggest that high levels of TRAIL-R4 decoy receptor expression should be accounted for when treating NSCLC patients with Ad5hTRAIL as the sole treatment modality.

In conclusion, this study demonstrates that surface TRAIL-R4 decoy receptor expression correlated well with the TRAIL resistance phenotype in lung cancer cells. Furthermore, in addition to NF-κB inhibition (Sanlioglu *et al*., 2006), the alteration of TRAIL receptor profiles of cancer cells by a DcR2 siRNA approach is a novel way of bypassing the TRAIL resistance encountered in cancer cells.

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Surface TRAIL decoy receptor-4 expression is correlated with TRAIL resistance in MCF7 breast cancer cells

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Abstract

Background: Tumor Necrosis Factor (TNF)-Related Apoptosis-Inducing Ligand (TRAIL) selectively induces apoptosis in cancer cells but not in normal cells. Despite this promising feature, TRAIL resistance observed in cancer cells seriously challenged the use of TRAIL as a death ligand in gene therapy. The current dispute concerns whether or not TRAIL receptor expression pattern is the primary determinant of TRAIL sensitivity in cancer cells. This study investigates TRAIL receptor expression pattern and its connection to TRAIL resistance in breast cancer cells. In addition, a DcR2 siRNA approach and a complementary gene therapy modality involving IKK inhibition (AdIKKβKA) were also tested to verify if these approaches could sensitize MCF7 breast cancer cells to adenovirus delivery of TRAIL (Ad5hTRAIL).

Methods: TRAIL sensitivity assays were conducted using Molecular Probe's Live/Dead Cellular Viability/ Cytotoxicity Kit following the infection of breast cancer cells with Ad5hTRAIL. The molecular mechanism of TRAIL induced cell death under the setting of IKK inhibition was revealed by Annexin V binding. Novel quantitative Real Time RT-PCR and flow cytometry analysis were performed to disclose TRAIL receptor composition in breast cancer cells.

Results: MCF7 but not MDA-MB-231 breast cancer cells displayed strong resistance to adenovirus delivery of TRAIL. Only the combinatorial use of Ad5hTRAIL and AdIKKβKA infection sensitized MCF7 breast cancer cells to TRAIL induced cell death. Moreover, novel quantitative Real Time RT-PCR assays suggested that while the level of TRAIL Decoy Receptor-4 (TRAIL-R4) expression was the highest in MCF7 cells, it was the lowest TRAIL receptor expressed in MDA-MB-231 cells. In addition, conventional flow cytometry analysis demonstrated that TRAIL resistant MCF7 cells exhibited substantial levels of TRAIL-R4 expression but not TRAIL decoy receptor-3 (TRAIL-R3) on surface. On the contrary, TRAIL sensitive MDA-MB-231 cells displayed very low levels of surface TRAIL-R4 expression. Furthermore, a DcR2 siRNA approach lowered TRAIL-R4 expression on surface and this sensitized MCF7 cells to TRAIL.

Conclusion: The expression of TRAIL-R4 decoy receptor appeared to be well correlated with TRAIL resistance encountered in breast cancer cells. Both adenovirus mediated IKKβKA expression and a DcR2 siRNA approach sensitized MCF7 breast cancer cells to TRAIL.

Background

Cancer still appears to be a challenging disease to treat. According to most recent estimates, more than 10 million new cancer cases were reported in the year 2000 killing around 6 million people [1]. In addition, 10 % of all cancers appear to be the breast cancer. Being the most frequently diagnosed cancer type in women, the breast cancer claims about 370,000 deaths each year around the world [2]. Surgery, radiotherapy and chemotherapy are among the most widely used treatment methods for patients with breast cancer [3-5]. Still, these conventional treatment modalities did not improve the survival rate of patients with locally advanced or metastatic breast cancer. With standard therapy, locally advanced breast cancer has a five year survival rate of 55 % and a ten year survival rate of 35 % [6]. There is a 40 % recurrence rate after ten years following the diagnosis and removal of primary tumor in patients with breast cancer [7]. For all these reasons, novel treatment methods are needed for the treatment of patients with breast cancer.

Induction of programmed cell death known as apoptosis [8], appears to be a viable alternative to currently employed treatment modalities in the fight against cancer [9]. In order for chemotherapy and radiotherapy treatment options to work as anticancer agents; tumor suppressor gene, p53, is required [10]. Unfortunately, p53 mutations are acquired during the progression of cancer in more than half of the human tumors [11,12]. Therefore, the resistance to both chemotherapy and radiotherapy is almost unavoidable in tumors lacking p53 [13]. On the other hand, death ligands are capable of inducing apoptosis independently of p53 status of cells [14]. Because of this reason, death ligands are currently considered as anticancer agents [15]. Among the death ligands tested, Tumor Necrosis Factor (TNF) [16-18] and FasL [19] effectively induced apoptosis in cancer cells. However, due to their systemic toxicity, the application of these agents in cancer gene therapy is very limited. The discovery of a novel death ligand, TRAIL [20,21], changed this view, since unlike other members of the TNF family, TRAIL selectively killed cancer cells without causing any harm to normal cells [22]. Thus, treating tumor cells with TRAIL ligand appeared as an invaluable way of inducing apoptosis specifically in tumor cells, as normal cells are protected against the death-inducing effects of TRAIL [23,24]. However, the mechanism of TRAIL resistance in normal cells is not understood [25] and significant proportions of cancer cells [26] including those of breast [27,28] appeared to be TRAIL resistant. Consequently, TRAIL resistance constitutes a barrier if one wishes to use TRAIL as a death ligand in any breast cancer gene therapy approach.

Resistance to TRAIL-induced apoptosis in normal cells was initially considered to be caused by the presence of decoy receptors (TRAIL-R3 and TRAIL-R4), which compete with death receptors (TRAIL-R1 and TRAIL-R2) for binding to TRAIL [29,30]. So far, no correlation between TRAIL sensitivity and the expression pattern of TRAIL receptors has been demonstrated in cancer cells yet [31]. The presence of intracellular apoptosis inhibitory substances (bcl-xL, c-FLIP, cIAP etc.) was also blamed to be responsible for TRAIL resistance [31-33]. Intriguingly, the engagement of both TRAIL death receptors and TRAIL-R4 decoy receptor also activated NF-kB pathway [24,34,35]. Because NF-kB activation is known to hamper the apoptotic pathways in cells by up-regulating the expression of various apoptosis inhibitory molecules such as cFLIP, bcl-xL, c-IAP and the decoy receptor TRAIL-R3 [34,36,37], high levels of NF-kB activation might be a strong factor responsible for blocking apoptotic processes in order to establish TRAIL resistance. For this reason, we analyzed both the TRAIL induced as well as endogenous NF-kB activities using Luciferase reporter gene assays in MCF7 breast cancer cells. Because TRAIL-R1, TRAIL-R2 and TRAIL-R4 induced NF-kB activation has been shown to be primarily mediated by TRAF2-NIK-IkappaB kinase alpha/ beta signaling cascade [35], MCF7 breast cancer cells were coinfected with adenovirus vectors encoding a dominant negative mutant to IKKβ(AdIKKβKA) [38] and hTRAIL (Ad5hTRAIL) in order to test if TRAIL resistance in breast cancer cells is eliminated through the inhibition of IKK, a leading modulator of NF-kB. The molecular mechanism of TRAIL resistance in breast cancer cells (MCF7 and MDA-MB-231) was studied by novel Real Time RT-PCR assays and conventional flow cytometry in order to verify if there is any relationship between TRAIL resistance and the expression pattern of TRAIL receptors. Lastly, a DcR2 siRNA approach was utilized to knock down the expression of relevant TRAIL decoy receptor in order to reveal its connection to TRAIL resistance.

Methods

Recombinant adenovirus vector production

Amplification of the vectors Ad5hTRAIL [39], AdIKKβKA [17], AdEGFP [18], AdCMVLacZ [40] and AdNFkBLuc [38] was performed as previously described [41]. Amplified vectors were stored at -80°C in 10 mM Tris with 20 % glycerol. AdIKKβKA expresses a dominant negative mutant of IKKβ, which interacts with other IKK subunits to form inactive IKK complexes. The particle titers of adenoviral stocks were in the range of 10^{13} DNA particles/ml, whereas the typical particle/plaque forming unit ratio was equal to 50.

Infection of breast cancer cells with first generation recombinant adenovirus vectors
Breast cancer cell lines were cultured in RPMI 1640 medium supplemented with 10 % FBS, 2.2 g/l sodium bicarbonate, 1 mM L-glutamine, and 1 % penicillin-streptomycin mixture, at 37°C in a humidified 5 % CO_2 atmosphere. Experimental steps of transduction of breast cancer cells with adenoviral vectors can be summarized as follows: Breast cancer cells were infected with an increasing multiplicity of infection (MOI) of AdEGFP (vector expressing enhanced green fluorescent protein (EGFP) reporter gene) vector at 37°C in RPMI 1640 without FBS. Two hours following infection, equal volume of RPMI 1640 supplemented with 20 % FBS was added to increase the serum concentration in the media to 10 %. 48 hours after the infection, the level of transduction was detected by examining of the percentage of GFP (+) cells under a fluorescent microscopy and subsequently by flow cytometry. Propidium iodide exclusion technique was used to determine the cell viability. Overexpression of hTRAIL was provided by Ad5hTRAIL infection. Cells were coinfected with adenovirus vectors encoding IKKβ dominant negative mutant (AdIKKβKA) and Ad5hTRAIL in order to block IKK activity thereby NF-kB activation. NF-kB promoter based Luciferase assay system was utilized to conduct NF-kB transcription activation assays using AdNFkBLuc construct. AdCMVLacZ vector was used as a control.

NF-kB directed transcription activation assays
AdNFkBLuc construct was utilized in order to determine the NF-kB activation status of MCF7 cells. AdNFkBLuc vector [38] possesses four tandem copies of the NF-kB consensus sequence fused to a TATA-like promoter from the herpes simplex virus-thymidine kinase gene driving the expression of a Luciferase reporter. Transcriptional induction mediated by NF-kB in the presence or absence of TRAIL was measured according to the manufacturer's protocol using the Luciferase assay system with Reporter Lysis Buffer (Promega, Inc.). All measurements of Luciferase activity expressed as relative light units were normalized against the protein concentration.

Cell viability assays
Discrimination of live cells from dead cells was performed using Live/Dead Cellular Viability/Cytotoxicity Kit from Molecular Probes (Eugene, OR). This assay is based on the use of Calsein AM and Ethidium homodimer-1 (EthD-1). Calsein AM is a fluorogenic substrate for intracellular calsein esterase. It is modified to a green fluorescent compound (calsein) by active esterase in live cells with intact membranes, thus serves as a marker for viable cells. Unharmed cell membranes do not allow EthD-1, a red fluorescent nucleic acid stain, to enter inside the cell. However, cells with damaged membrane uptake the dye and stain positive.

Apoptosis detection by Annexin V binding
Annexin V conjugated to fluorochromes such as FITC has successfully been used as probes to detect cells undergoing apoptosis. Annexin V binding assays were carried out according to manufacturer's instructions (Alexis Biochemicals). For this purpose, a FITC conjugated mouse monoclonal antibody to human Annexin V (ALX-804-100F-T100) was employed to detect apoptotic cells via flow cytometry.

The detection of TRAIL receptor expression profile by flow cytometry
Anti-TRAIL receptor flow cytometry set (Cat. ALX-850-273-KI01) was used to detect TRAIL receptor protein expression on cell surface. This kit contains 100μgs of MAb to TRAIL-R1 (clone HS101, Cat. 804-297A), -R2 (clone HS201, Cat.804-298A), -R3 (clone HS301, Cat. 804-344A) and -R4 (clone HS402, Cat. 804-299A). Primary antibodies were used at 5 μg/ml concentration. Biotinylated goat anti-mouse IgG1 (Cat. ALX-211-202) was used as a secondary antibody followed by streptavidin-PE (Cat. ANC-253-050) prior to flow cytometry. Flow analysis was performed according to manufacturer's protocols using BD FACSCALIBUR at the Akdeniz University Hospitals. Purified mouse IgG1 (MOPC 31C, Cat. ANC-278-010) served as an isotype control.

Quantitative Real Time RT-PCR assay for human TRAIL receptors
TRIzol reagent (Life Technologies, Gaithersburg, MD) was used to extract total RNA from breast cancer cells, according to the instructions from the manufacturer. Reverse transcription of 2 μg of total RNA was performed using TaqMan Reverse Transcription Reagents (Applied Biosystems Cat. N8080234). Despite the fact that the sequences for TRAIL-R1 and TRAIL-R2 primers and probes were recently described by our group [42], we had to design new probe sets for the decoy receptors. Following is the sequence information for TRAIL decoy receptor sets: **TRAILR3-5'** CCC-TAA-AGT-TCG-TCG-TCG-TCA-T, **TRAILR3-3'** GGG-CAG-TGG-TGG-CAG-AGT-A, **TRAILR3 Probe: 5'** 6FAM-TCGCGGTCCTGCTGCCAGTCCTAGC-TAMRA 3'; **TRAILR4-5'** ACA-GAG-GCG-CAG-CCT-CAA, **TRAILR4-3'** ACG-GGT-TAC-AGG-CTC-CAG-TAT-ATT, **TRAILR4 Probe: 5'** 6FAM-AGGAGGAGTGTCCAGCAG-GATCTCATAGATC-TAMRA 3'. rRNA was amplified as an internal control in the same reaction. Both the rRNA primers and probes were obtained from PE Applied Biosystems (Cat. 4308329). ΔΔCt method was used as described by Applied Biosystems to calculate the relative quantities of TRAIL receptors. The TaqMan PCR reaction

was performed as described by the manufacturer (Applied Biosystems Cat. N8080228).

A DcR2 siRNA approach targeting TRAIL-R4 expression

Posttranscriptional silencing of gene expression became a very useful approach within the last couple of years in research. DcR2 siRNA experiments were performed using DcR2 siRNA (sc-35185), siRNA transfection medium (sc-36868) and siRNA transfection reagent (sc-29528) in MCF7 breast cancer cells as described by the manufacturer (Santa Cruz Biotechnology). Flow cytometry analysis was performed to assess any changes in TRAIL-R4 gene expression. MCF7 cells were infected with Ad5hTRAIL or AdCMVLacZ vectors at increasing doses 35 hours following the transfection. Molecular Probe's Live/Dead Cellular Viability/Cytotoxicity Kit was used to assess the amount of live cells 48 hours following the infection.

Results

MCF7 breast carcinoma cells were efficiently transduced with recombinant adenoviruses

In order to find out the efficacy of transduction of breast cancer cells by first generation adenoviral vectors, MCF7 cells were infected with increasing Multiplicity of Infection (MOI) of adenovirus encoding Enhanced Green Fluorescent Protein (AdEGFP). The transduction profiles were followed under fluorescent microscopy and the results were quantitatively analyzed by flow cytometry 48 hours following the infection (Figure 1). While an MOI of 5000 DNA particles/cell was sufficient to transduce more than 90 % of the cells, nearly 100 % of the cells were transduced with AdEGFP at an MOI of 10,000 DNA particles/cell. These assays were also pivotal in obtaining the optimum dose of adenovirus required for efficient transduction of MCF7 breast carcinoma cell line without observing deleterious cytotoxic effects. These results demonstrated that breast cancer cells were transduced successfully with recombinant adenoviral vectors.

MCF7 breast cancer cells displayed complete resistance to TRAIL

Although TRAIL appeared as a promising therapeutic ligand to treat cancer, a variety of tumor types were reported to be resistant to TRAIL-induced cell death. For this reason, we wanted to investigate if exogenous TRAIL expression delivered by adenovirus vectors would induce killing of breast cancer cells. To test this, MCF7 cells were infected with increasing titers of Ad5hTRAIL or AdCMVLacZ. Amount of viable cells were detected using Molecular Probe's Live/Dead Cellular Viability/Cytotoxicity Kit 48 hours following the infections (Figure 2). MCF7 cells displayed complete resistance to TRAIL, as no reduction in the level of viable cells was observed even at an MOI of 10,000 DNA particles/cell, at which almost all cells were infected. Thus, it was concluded that MCF7 breast cancer cells were completely resistant to adenovirus delivery of TRAIL. Similarly, AdCMVLacZ infection alone revealed no significant degree of cell death either (data not shown).

Blocking IKK induced NF-kB activation pathway alone did not cause any reduction in the viability of MCF7 breast carcinoma cells

Because increased NF-kB activity was claimed to be responsible for the resistance to death ligand induced cytotoxicity in some tumors [36,37], we wanted to test if the inhibition of IKK activity thereby NF-kB would reduce the viability of breast cancer cells. In order to block the intracellular anti-apoptotic NF-kB pathway, MCF7 cells were infected with increasing MOIs of adenoviral vectors encoding a dominant negative mutant of IKKβ(AdIKKβKA), a key molecule involved in the activation of NF-kB. Cell viability was examined 48 hours following the infection under fluorescent microscope (Figure 2). Interestingly, AdIKKβKA vector alone proved inefficient in reducing the viability of MCF7 cells, even at an MOI of 10,000 DNA particles/cell.

Adenovirus delivery of IKKβKA gene expression sensitized MCF7 breast cancer cells to TRAIL-induced apoptosis

Adenovirus-mediated delivery of IKKβ (Ad.IKKβKA) [17,18] or IkBα (Ad.IkBαSR) [40,43] dominant negative mutants have previously been demonstrated to sensitize lung cancer cells to TNF death ligand. Because most of the breast cancer cell lines tested appeared to be TRAIL resistant [27,28], NF-kB targeting strategies involving IKK inhibition was employed to verify whether MCF7 breast carcinoma cells were sensitized to TRAIL under these circumstances. To accomplish this, MCF7 cells were coinfected with a constant MOI of Ad5hTRAIL construct and increasing doses of AdIKKβKA vector. In order to better assess the sensitization phenomenon, Ad5hTRAIL was infected at two different MOIs into MCF7 breast cancer cell lines. While a constant MOI of 1000 DNA particles/cell of Ad5hTRAIL was used in infection experiments depicted on Figure 3, infection experiments conducted at an MOI of 5000 DNA particles/cell are displayed in Figure 4. The amount of viable cells was detected 48 hours following the infections using Molecular Probe's Live/Dead Cellular Viability/Cytotoxicity Kit. Intriguingly, MCF7 cells were sensitized to TRAIL only when Ad5hTRAIL was coinfected with AdIKKβKA vector. For instance, nearly 55 % cell death was observed when cells were coinfected with 1000 MOI of Ad5hTRAIL and 5000 MOI of AdIKKβKA constructs (Figure 3). When MOI of Ad5hTRAIL was increased to 5000 as depicted on Figure 4, the death rate went up to 90 %. On the other hand, AdCMVLacZ infection instead of AdIKKβKA in breast cancer cells revealed no TRAIL sensitization (data not shown). These results suggested that IKKβKA expression via adenoviral vectors

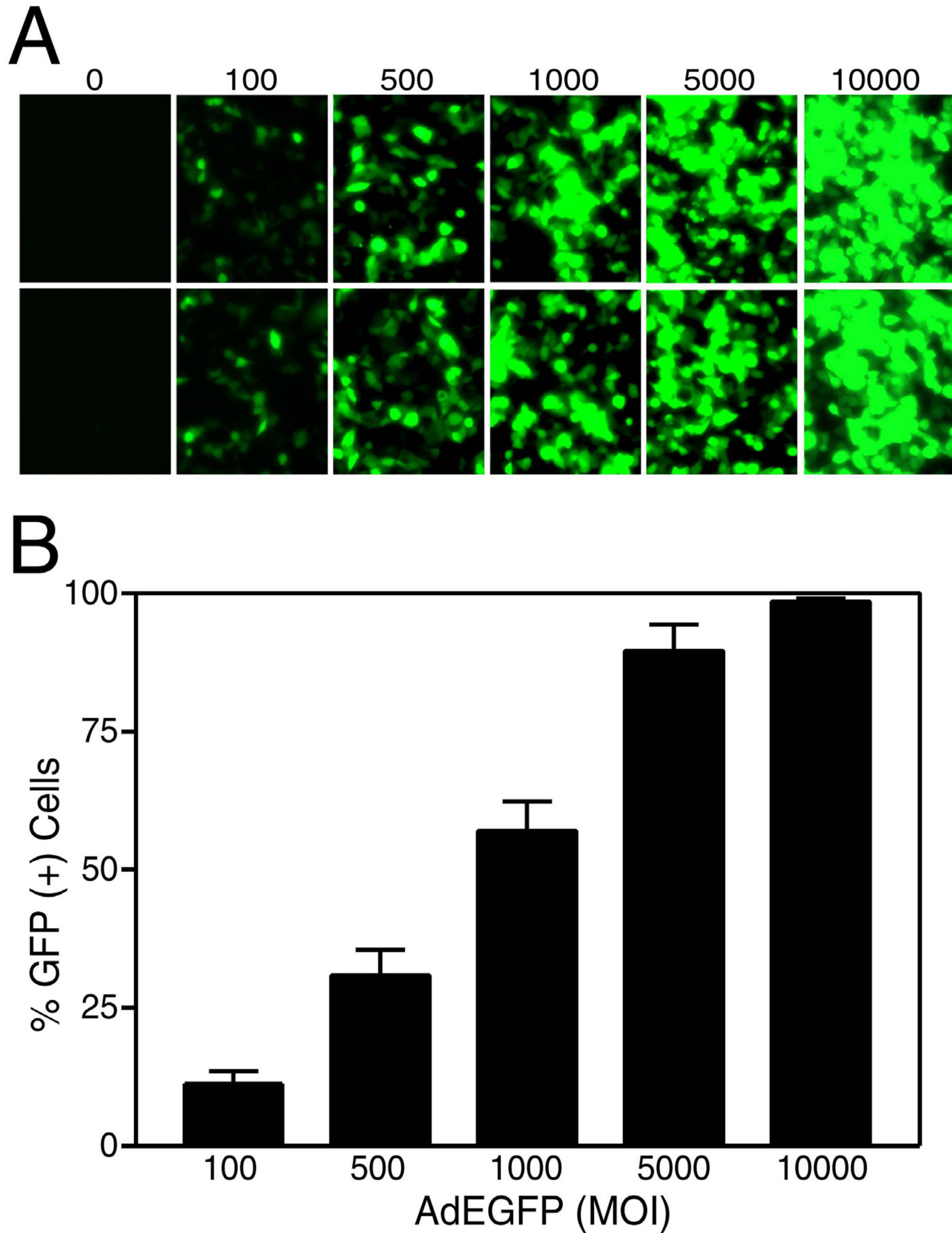


Figure 1
First generation adenoviral vectors efficiently transduced MCF7 breast cancer cells. MCF7 cells were infected with increasing MOIs of AdEGFP for 48 hours prior to analysis. The number of EGFP expressing cells was detected under fluorescent microscopy (Panel A), and analyzed by flow cytometry (Panel B). Numbers represent viral doses applied in MOI values as DNA particles/cell.

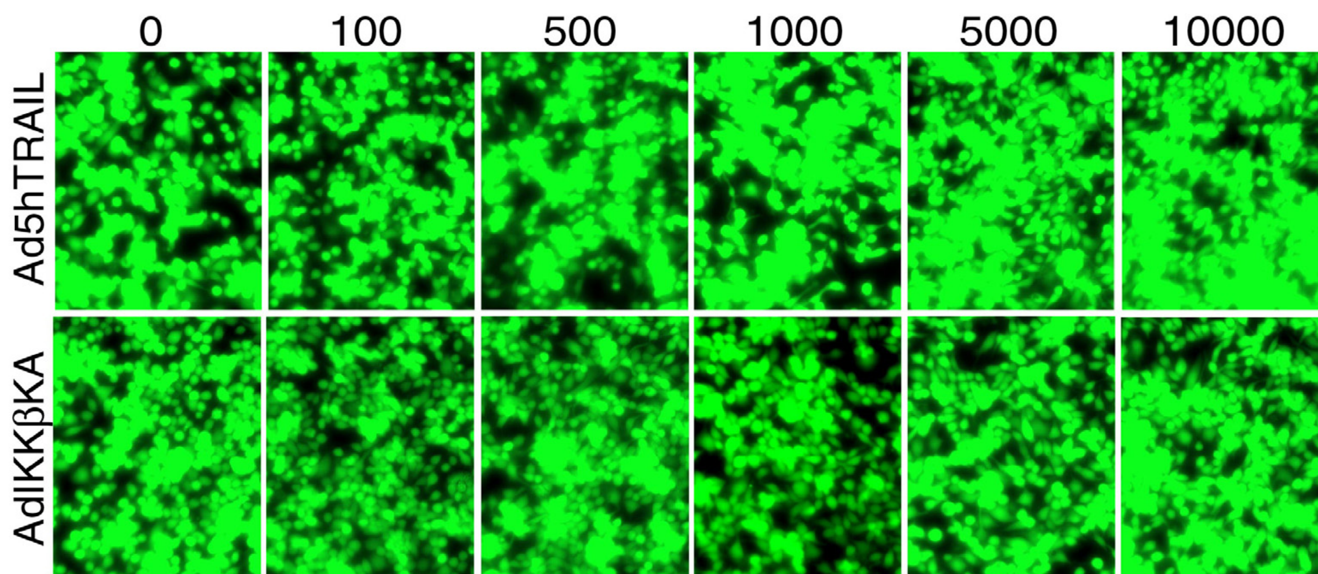


Figure 2
Ad5hTRAIL or AdIKKβKA infection alone did not decrease the viability of MCF7 breast cancer cells. MCF7 cells were infected with increasing MOIs of either Ad5hTRAIL or AdIKKβKA construct. Cell viability was detected using Molecular Probe's Live/Dead Cellular Viability/Cytotoxicity Kit 48 hours following the infection. Numbers represent viral doses applied, in MOI values as DNA particles/cell.

defeated TRAIL resistance observed in MCF7 breast cancer cells.

Exogenous TRAIL overexpression elevated the basal NF-kB activity in MCF7 cells, whereas IKKβKA expression blocked both TRAIL-induced and basal NF-kB activities

It is well known that different tumor cells display diverse levels of endogenous NF-kB activities. Furthermore, intracellular NF-kB activity in tumor cells is upregulated by both TRAIL death receptors (TRAIL-R1 and TRAIL-R2) [34,44] as well as TRAIL decoy receptor TRAIL-R4 [45] upon ligand binding. Knowing the endogenous NF-kB status of cancer cells before the therapy is obviously crucial for TRAIL mediated gene therapy targeting to induce apoptosis in cancer cells. A coinfection experiment was performed using a recombinant adenovirus vector carrying NF-kB driven Luciferase reporter gene (AdNFkBLuc) and Ad5hTRAIL vector in order to study the extent of NF-kB activation as a result of TRAIL overexpression in MCF7 breast cancer cell line. NF-kB Luciferase assays were conducted 24 hours following the infection in order to determine cell's NF-kB activation status. As seen in Figure 5, Ad5hTRAIL at an MOI of 5000 DNA particles/cell (Panel B) but not at an MOI of 1000 DNA particles/cell (Panel A) stimulated NF-kB activation. In order to determine the magnitude of NF-kB inhibition, a triple coinfection experiment involving AdNFkBLuc, Ad5hTRAIL and AdIKKβKA or AdCMVLacZ was performed. While IKKβKA overexpression in MCF7 cells gradually reduced both the TRAIL-induced and basal NF-kB activities in MCF7 cells, no such NF-kB inhibiting effect was observed in cells upon superinfection with AdCMVLacZ virus as a control (Figure 5).

Coinfection of Ad5hTRAIL and AdIKKβKA results in apoptotic cell death in MCF7 breast cancer cells

To show that apoptosis is the mechanism of cell death mediated by TRAIL overexpression under the setting of IKK inhibition in MCF7 cells, Annexin V staining was performed using flow cytometry. For this purpose, MCF7 cells were infected with Ad5hTRAIL or AdIKKβKA vectors alone or in combination. Thirty-five hours following the infection, apoptotic cell death was analyzed by Annexin-V-FITC staining. As displayed in Figure 6 Panel A, there was no substantial Annexin V binding generated by the expression of TRAIL or IKKβKA in MCF7 cells. However, considerable levels of Annexin V binding were observed in cells coinfected with Ad5hTRAIL and AdIKKβKA indicating apoptotic cell death (Figure 6, Panel B). As predicted, Ad5hTRAIL and AdCMVLacZ (negative control) coinfection did not yield any significant levels of Annexin V binding as MCF7 cells are resistant to TRAIL in the absence of IKK inhibition. These results suggested that the

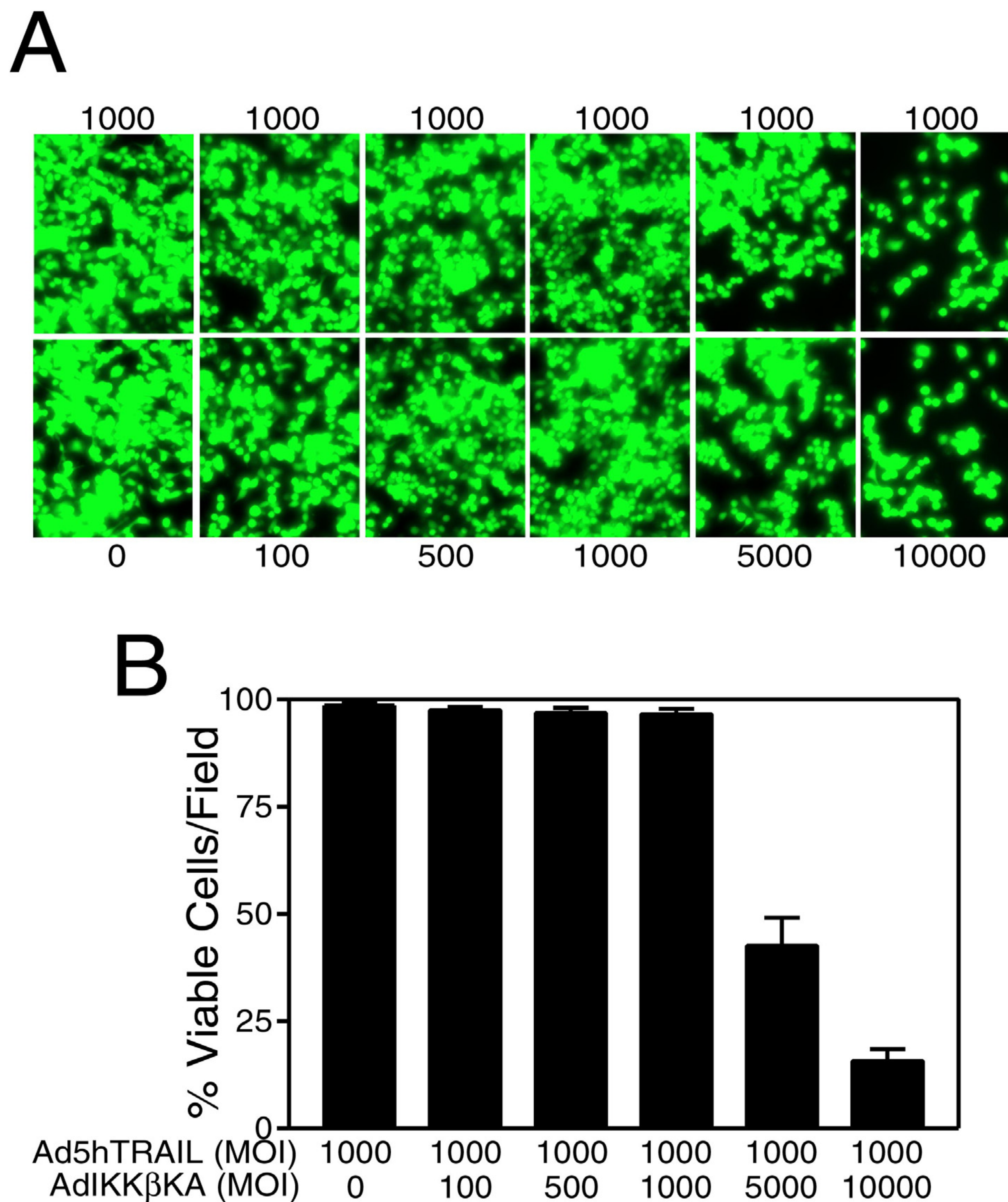
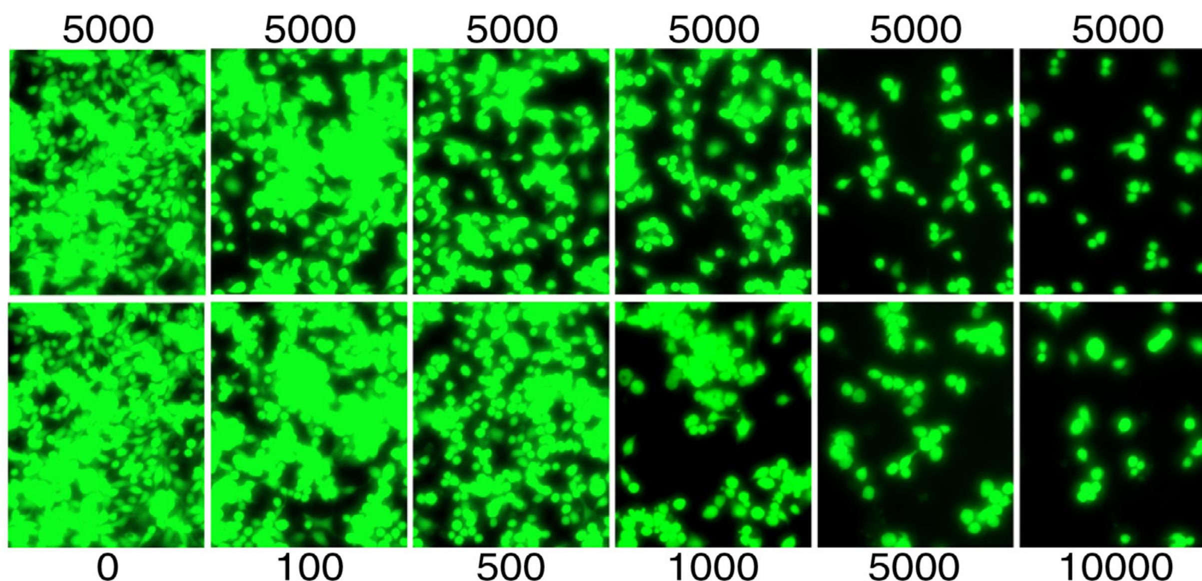


Figure 3
IKKβKA expression via adenoviral vectors sensitized MCF7 cells to TRAIL-mediated apoptosis. MCF7 cells were infected with increasing doses of adenoviral vectors encoding dominant negative mutant of IKKβ (as shown below each panel), while simultaneous infection with Ad5hTRAIL (as shown above each panel) was performed at a constant MOI of 1000. Cell viability was detected using Molecular Probe's Live/Dead Cellular Viability/Cytotoxicity Kit 48 hours following infection. Numbers represent viral doses applied in MOI values as DNA particles/cell. Fluorescent micrographs are provided in Panel A; Panel B depicts quantitative analysis of such infections. Values represent the mean (± SEM) of three different experiments.



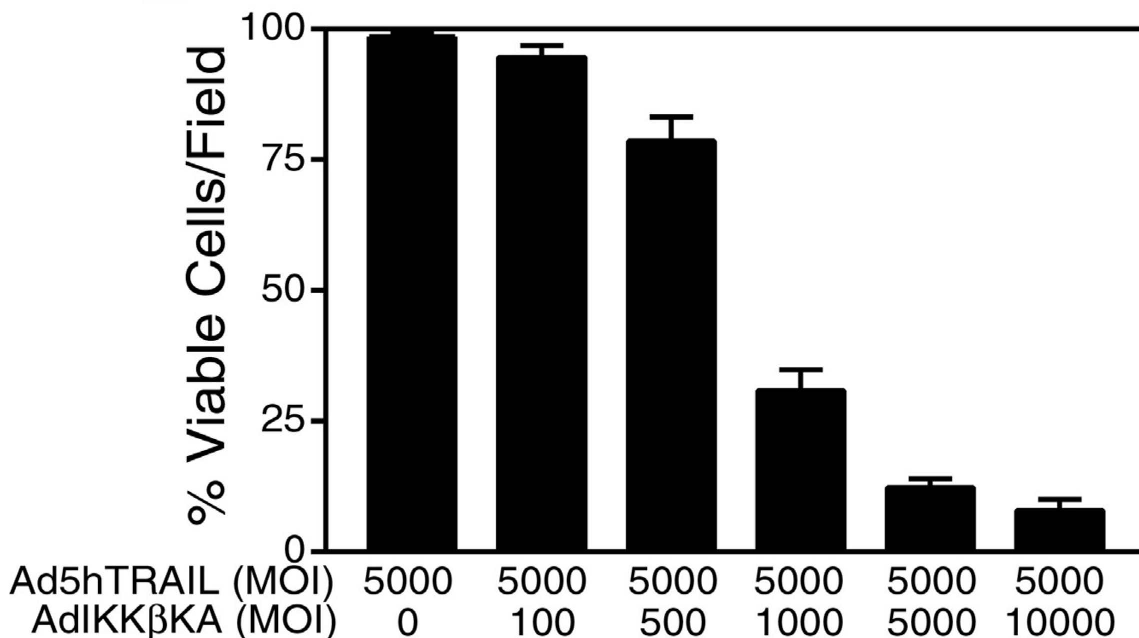


Figure 4
AdIKKβKA infection defeated the resistance to TRAIL-induced apoptosis in MCF7 breast cancer cells. These cells were coinfected with a constant MOI of 5000 DNA particles/cell of Ad5hTRAIL (as shown above each panel) and increasing doses of AdIKKβKA (as shown below each panel). Live/Dead Cellular Viability/Cytotoxicity Kit from Molecular Probe was used to detect TRAIL cytotoxicity 48 hours following infection. Numbers represent viral doses applied, in MOI values as DNA particles/cell. Data represent the mean of (± SEM) six independent data points (n = 6).

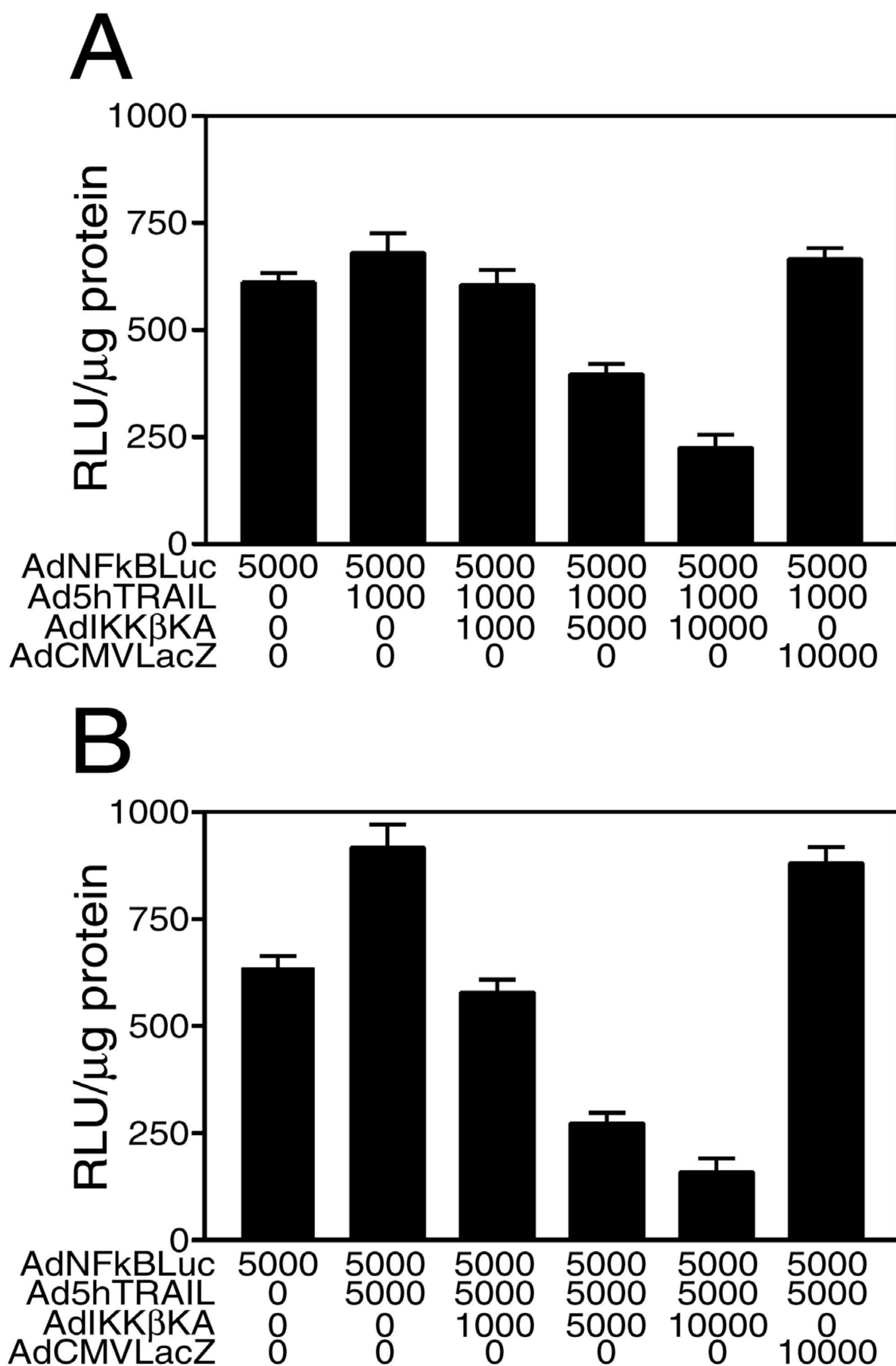


Figure 5

Distinctive regulation of NF-kB activation in MCF7 breast cancer cells by Ad5hTRAIL and/or AdIKKβKA infections. MCF7 cells were simultaneously infected with AdNFkBLuc, Ad5hTRAIL and/or increasing doses of AdIKKβKA construct for 24 hours. AdCMVLacZ infection was also performed as a negative control. The types of constructs used in the infection are shown on the x axis. MOI values represent DNA particles/cell. Ad5hTRAIL vector was used at two different constant MOIs (MOI of 1000 and 5000) in order to avoid cell death complicating our assay result. Luciferase activity expressed in Relative Light Units per microgram protein is shown on y axis. Values represent the mean (± SEM) of six independent data points (n = 6).

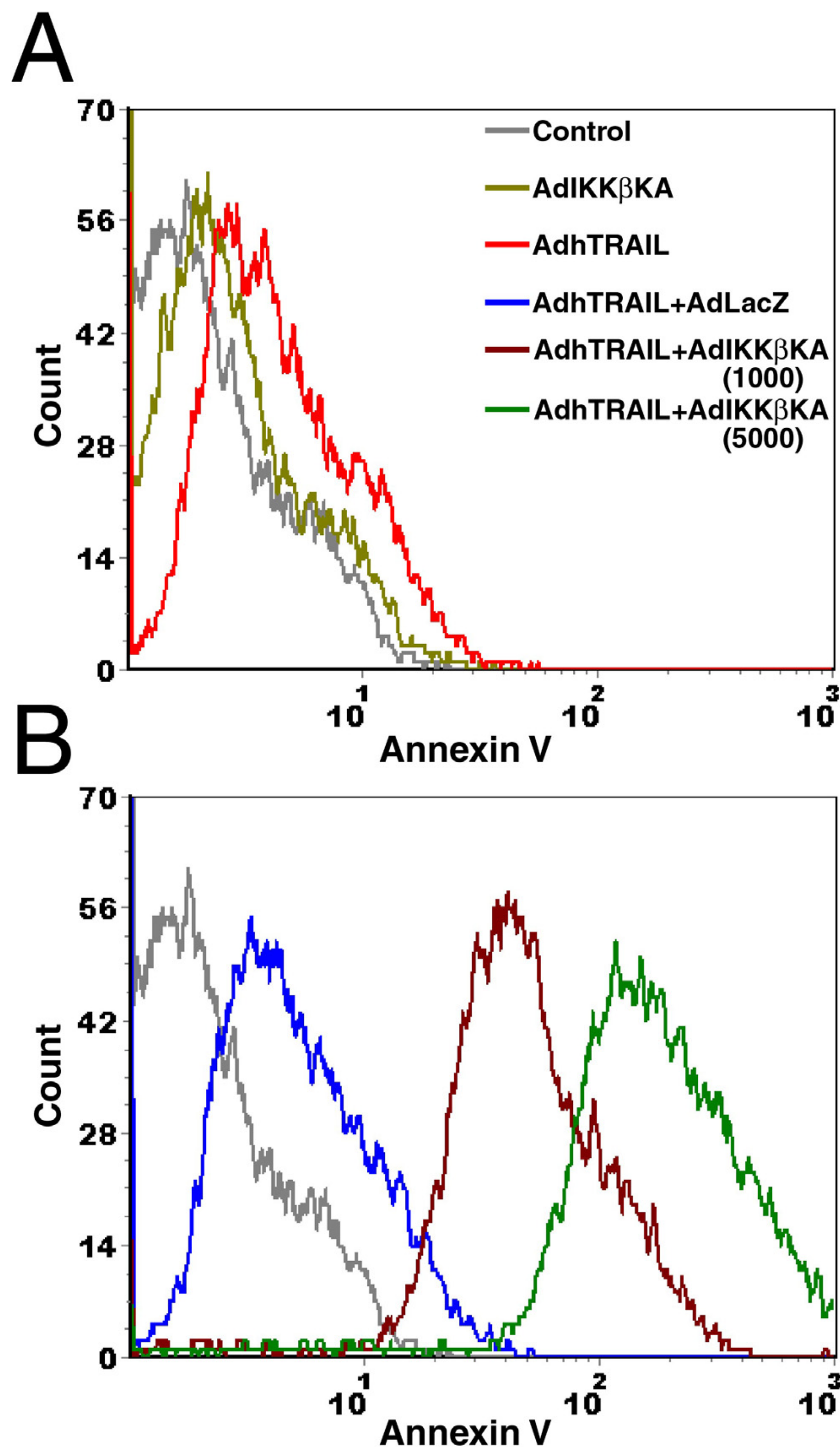


Figure 6
Ad5hTRAIL and AdIKKβKA coinfection induced apoptosis in MCF7 breast carcinoma cells. FITC conjugated Annexin V and Propidium Iodide (PI) staining were utilized using MCF7 cells infected with various combinations of adenovirus constructs as described in Methods prior to flow cytometry. Each histogram represents 10^4 gated MCF7 cells. Histograms were illustrated in two panels for clarity. Various treatment settings were provided in Panel A. MOI of 5000 DNA particles/cell was used for each viral construct unless stated otherwise in the Figure. Control line represents uninfected but FITC-Annexin V and PI stained MCF7 cells. Only one representative assay out of three independent assays was provided.

mechanism of cell death experienced by MCF7 cells is apoptosis following TRAIL stimulation under the setting of IKK inhibition.

MCF7 breast cancer cell line displayed significant levels of TRAIL decoy receptor-4 expression

So far no evidence of the connection between the expression pattern of TRAIL receptors and TRAIL sensitivity was found in cancer cells [31]. Part of the reason might have been the inability to screen all TRAIL receptors at once in breast cancer cells then [28]. In order to compensate this deficiency, quantitative novel Real Time RT-PCR assays were conducted using primer-probe sets specifically designed to detect each TRAIL receptor in MCF7 breast cancer cells (Figure 7, Panel A). According to our results, while all TRAIL receptors were expressed in MCF7 cells, TRAIL-R4 expression was the highest among the four. In addition, the level of TRAIL-R2 expression was much higher than that of TRAIL-R1. Lastly, TRAIL-R3 decoy receptor expression was the lowest. These results suggested that high levels of TRAIL-R4 decoy receptor expression correlated well with TRAIL resistance. However, as the gene expression detected inside the cell may not necessarily correlate with the receptor expression on cell surface, we decided to perform flow cytometry analysis using antibodies specific to four different TRAIL receptors. As shown in Figure 7 Panel B, MCF7 cells expressed all TRAIL receptors excluding TRAIL-R3 on cell surface. While similar levels of TRAIL death receptors TRAIL-R1 and TRAIL-R2 were expressed, there were still considerable levels of TRAIL-R4 decoy receptor expression on the surface of MCF7 cells.

TRAIL sensitive MDA-MB-231 cells displayed very low levels of TRAIL-R4 decoy receptor expression on cell surface

In order to solidify the importance of TRAIL-R4 expression and its connection to TRAIL resistance, another breast cancer cell line, MDA-MB-231, was also analyzed in terms of TRAIL receptor expression profile. Real Time RT-PCR assays revealed that while TRAIL-R2 expression was the highest on transcript levels, TRAIL-R4 decoy receptor expression was the lowest TRAIL receptor expressed in MDA-MB-231 breast cancer cells (Figure 8, Panel A). Furthermore, flow cytometry analysis indicated that insignificant levels of TRAIL-R4 expression were detected on the surface of MDA-MB-231 breast cancer cells (Figure 8, Panel B). TRAIL-R3 decoy receptor expression, however, was not detectable using flow cytometry. Intriguingly, in contrast to what was observed with MCF7, adenovirus delivery of TRAIL alone killed significant proportions of MDA-MB-231 breast cancer cells (Figure 9).

Lowering of TRAIL-R4 gene expression sensitized MCF7 breast cancer cells to TRAIL

In order to solidify the connection between TRAIL-R4 decoy receptor gene expression and TRAIL resistance, a DcR2 siRNA approach was executed in TRAIL resistant MCF7 breast cancer cells. Flow cytometry analysis conducted 35 hours following the transfection revealed that the level of TRAIL-R4 protein expression on surface went down drastically (Figure 10, Panel A). At this stage, MCF7 cells were further infected with either Ad5hTRAIL or AdCMVLacZ vector at increasing doses. Cell viability assays were conducted 48 hours following the infection. Only Ad5hTRAIL infected cells exhibited considerable amount of cell death following transfection (Figure 10, Panel B). No such effect was observed when cells were infected with AdCMVLacZ virus (data not shown).

Discussion

Although, conventional treatment modalities could not satisfactorily improve the survival rates of patients with locally advanced and metastatic disease, adenovirus delivery of death ligands represents a feasible choice for the treatment of patients with breast cancer. However, recent observations demonstrating that a considerable portion of human cancers including those of the breast [27,28] were TRAIL resistant undermined the potential application of TRAIL against cancer. Accordingly, the understanding of the mechanism of TRAIL resistance is the key to resolve primary obstacles in TRAIL mediated gene therapy approach. Based on recent findings from our laboratory and others, we think that NF-kB signaling is one of the most crucial pathways involved in the constitution of TRAIL resistance [26]. Despite the fact that TRAIL-R1, TRAIL-R2 and TRAIL-R4 induced NF-kB activation has been shown to be primarily mediated by TRAF2-NIK-IkappaB kinase alpha/beta signaling cascade [35], there is some doubt on whether or not NF-kB activation can block TRAIL mediated apoptosis. For example, in one particular study it was reported that NF-kB inhibition by way of IkappaBalpha mutant expression sensitized MCF7 cells to TNF but not TRAIL-induced apoptosis [35]. Considering the fact that there are different ways to activate NF-kB pathway (IkB dependent and independent ways) [46] we decided to inhibit IKK activity rather than targeting IkappaBalpha itself to look for the possibility of sensitizing MCF7 breast cancer cells to TRAIL.

First of all, in order to find out the efficacy of adenovirus transduction in breast cancer cells, MCF7 cells were infected with increasing MOIs of AdEGFP virus. The transduction profiles analyzed by flow cytometry showed that nearly 100 % of the cells were transduced with AdEGFP at an MOI of 10,000 DNA particles/cell (Figure 1). The efficacy of TRAIL in mediating apoptosis of MCF7 breast cancer cells was assessed using Ad5hTRAIL

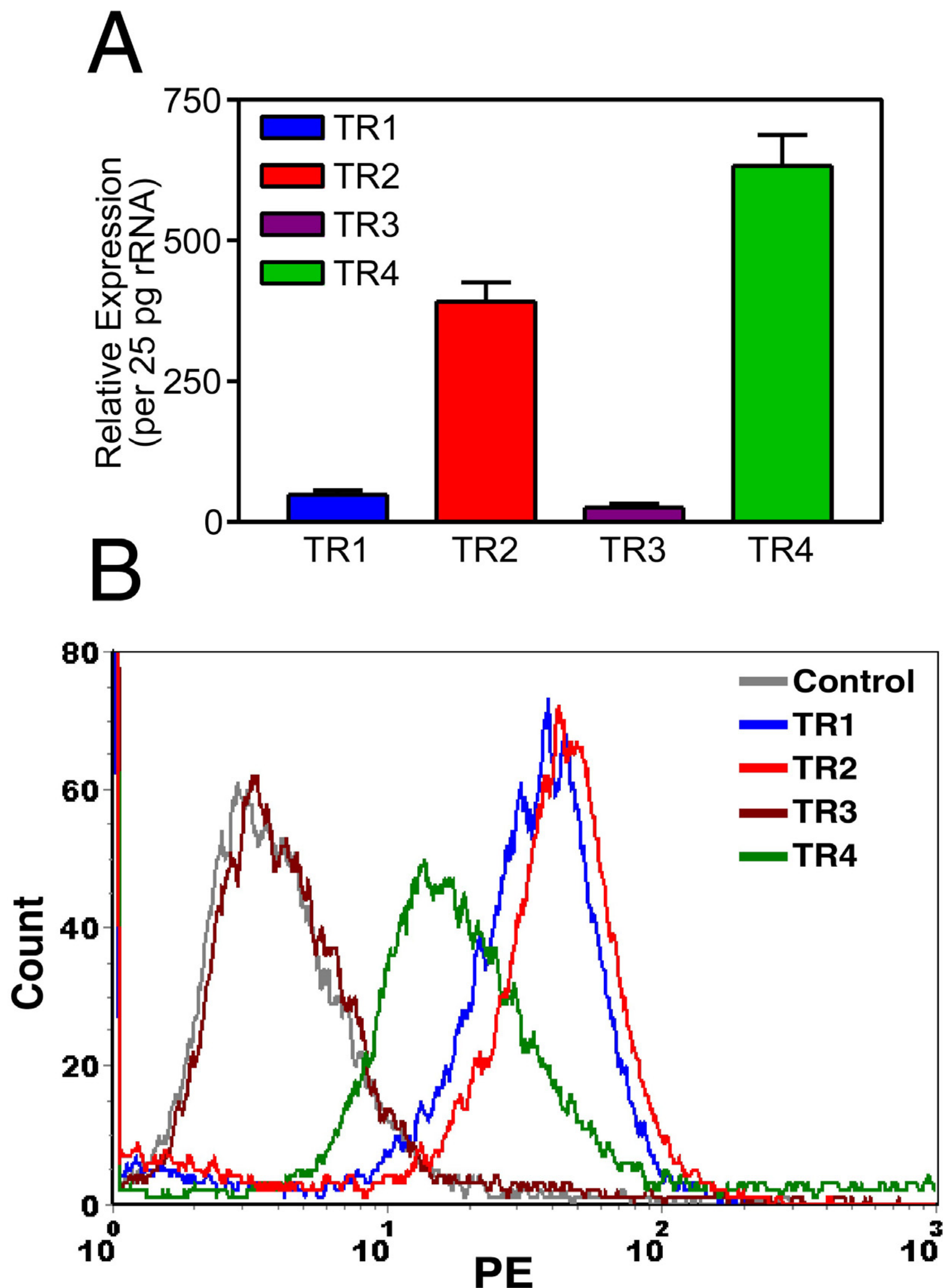


Figure 7
MCF7 breast carcinoma cell line displayed substantial levels of TRAIL-R4 decoy receptor expression. Quantitative Real Time RT-PCR of TRAIL receptors was performed as described in Methods (Panel A). TRAIL receptor levels per 25 pg of ribosomal cDNA are presented in the graph for clarity. Ribosomal RNA primers and probes were included in each TaqMan reaction as an internal control. Panel B depicts the surface TRAIL receptor expression pattern of MCF7 cells using flow cytometry. Experimental parameters are defined in colored lines. 10^4 cells were gated for each histogram. Only one representative assay for each experiment (independently repeated three times) is shown.

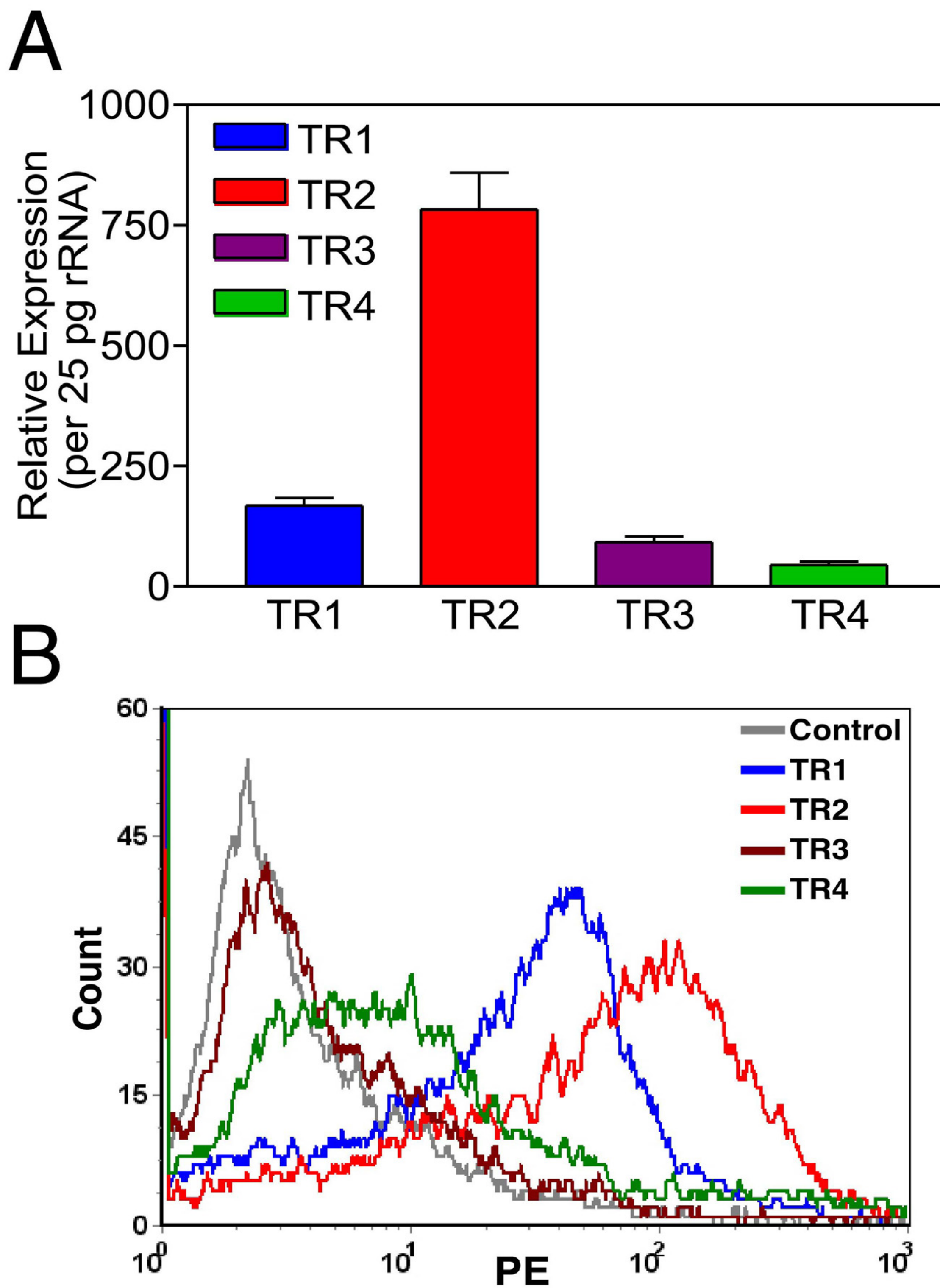


Figure 8
MDA-MB-231 breast cancer cells displayed trivial levels of TRAIL-R4 decoy receptor expression on surface. TRAIL receptor composition of MDA-MB-231 breast cancer cells revealed by Real Time RT-PCR assay is displayed in Panel A. Panel B illustrates flow cytometry analysis showing the surface expression pattern of TRAIL receptors. 10^4 cells were gated for each histogram. Only one representative assay out of three is shown.

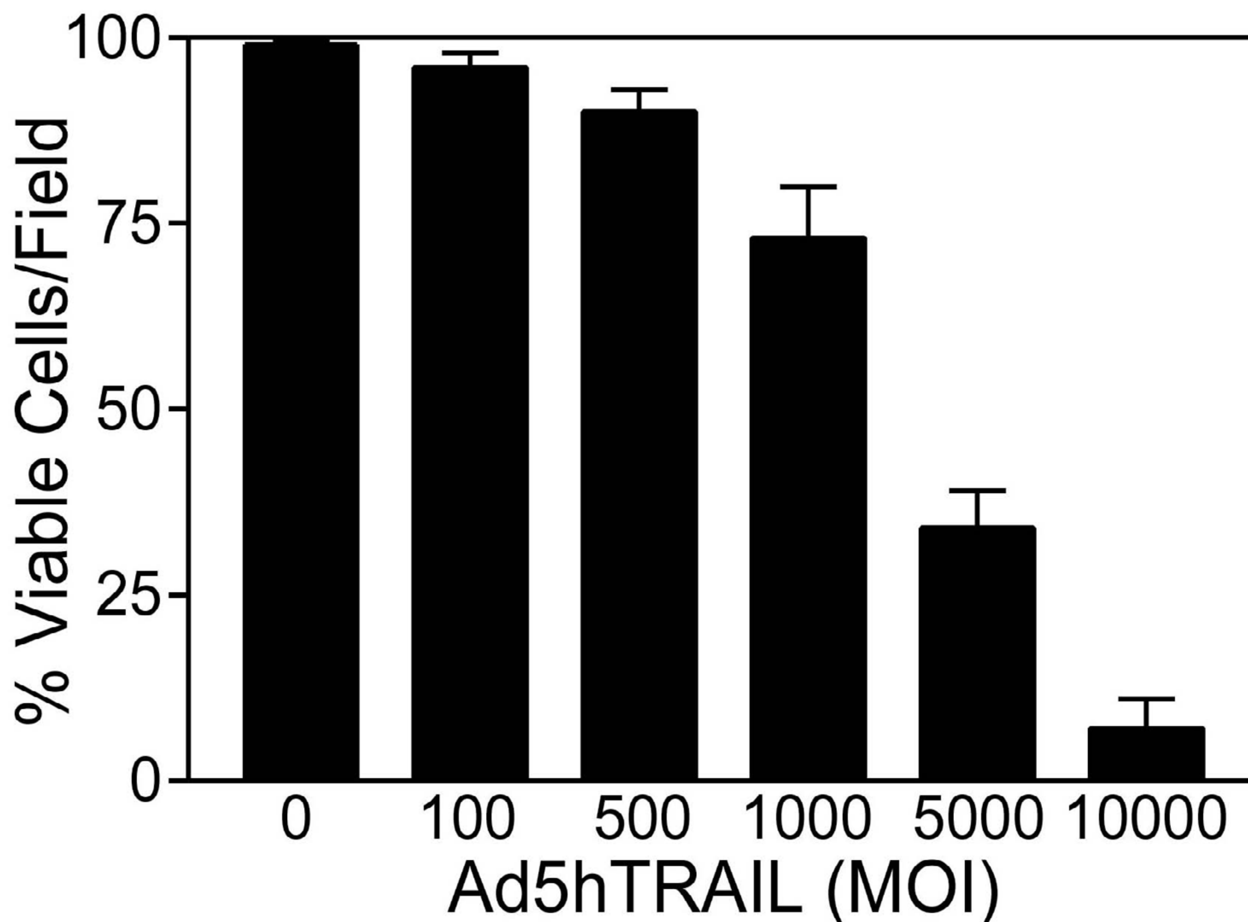


Figure 9
MDA-MB-231 breast cancer cell line is sensitive to Ad5hTRAIL infection. MDA-MB-231 breast cancer cells were infected with increasing MOIs of Ad5hTRAIL construct. Molecular Probe's Live/Dead Cellular Viability/Cytotoxicity Kit was used to detect % viable cells 48 hours following the infection. Numbers represent viral doses applied in MOI values as DNA particles/cell. Values represent the mean (± SEM) of six independent data points (n = 6).

construct. Interestingly, MCF7 cells displayed complete resistance to TRAIL as no reduction in the level of viable cells was observed even at an MOI of 10,000 DNA particles/cell (Figure 2). IKK inhibiting strategy alone proved inefficient in reducing the viability of MCF7 cells suggesting that an apoptotic stimulus was required in order to induce cell killing (Figure 2). Interestingly, in order to break down TRAIL resistance and to induce cell death, a coinfection of MCF7 cells with Ad5hTRAIL and AdIKKβKA was required (Figures 3 and 4). Luciferase assays confirmed that both the TRAIL induced and endogenous NF-kB activities were drastically reduced by the infection of MCF7 cells with AdIKKβKA virus (Figure 5). Moreover, IKKβKA sensitization of MCF7 breast carcinoma cells resulted in TRAIL induced apoptosis as revealed by Annexin V binding assays (Figure 6). These results suggested that NF-kB activation pathway has a hampering effect on TRAIL-induced cell death in MCF7 cells, and blocking this pathway is essential to sensitize breast cancer cells to TRAIL mediated apoptosis.

So far, no correlation between TRAIL resistance and TRAIL decoy receptor gene expression has been reported. For example, analysis of breast cancer cell lines by just examining the expression levels of TRAIL death receptors (TRAIL-R1 and TRAIL-R2) and TRAIL-R3 decoy receptor using RNase protection assay did not reveal any connection between the expression pattern of TRAIL receptors and TRAIL resistance [28]. But whether or not TRAIL-R4 decoy receptor gene expression in any way contributes to

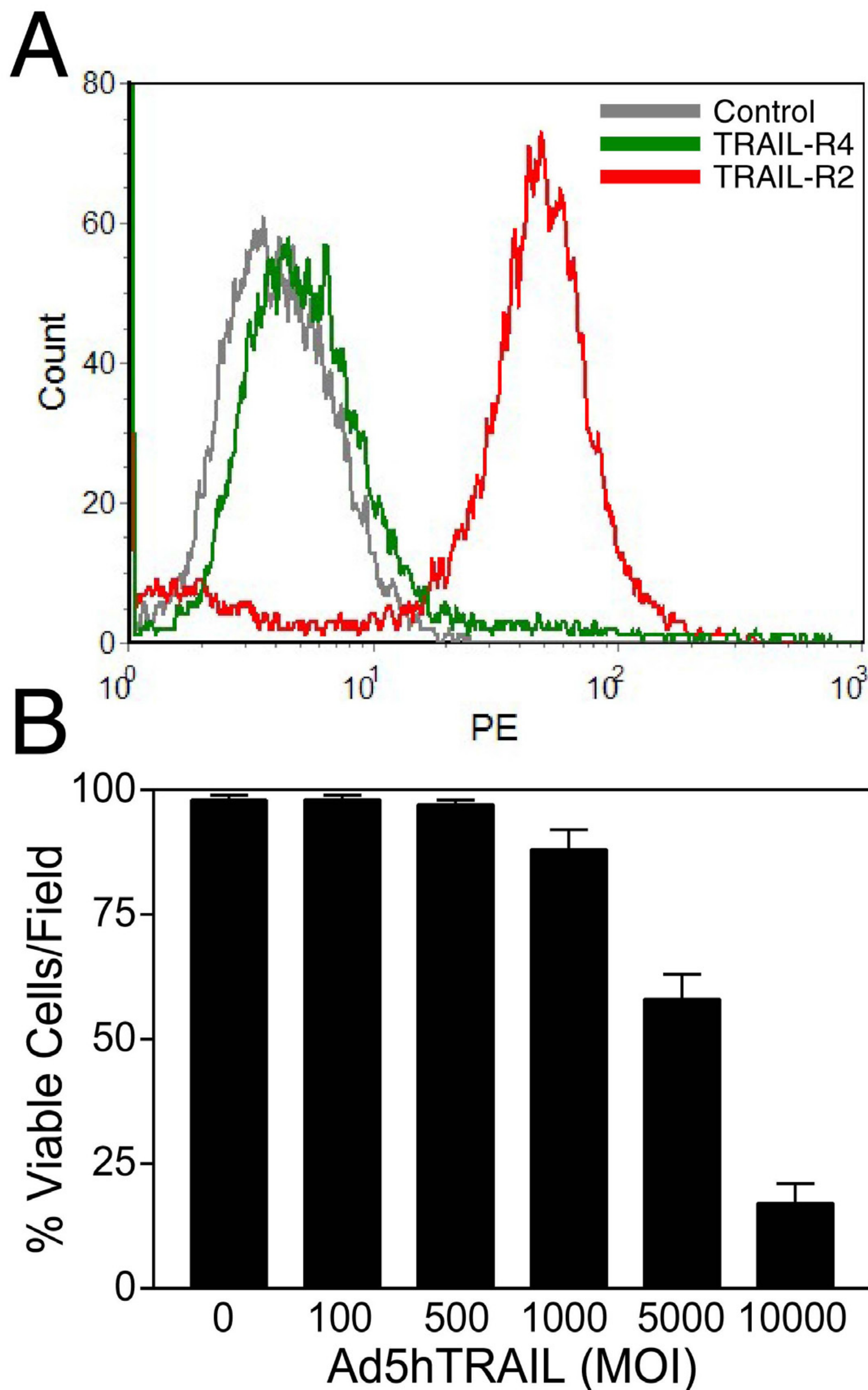


Figure 10

Knocking down TRAIL-R4 expression sensitized MCF7 breast cancer cells to TRAIL. A DcR2 siRNA approach was administered as described in Methods using TRAIL resistant MCF7 breast cancer cell line. Panel A depicts a flow cytometry analysis confirming strong attenuation of TRAIL-R4 expression on cell surface. TRAIL-R2 death receptor expression was also detected as a control. Sensitization of MCF7 breast cancer cells to TRAIL following a DcR2 siRNA approach is provided in Panel B. MCF7 breast cancer cells were infected with increasing doses of Ad5hTRAIL alone following a DcR2 siRNA transfection. Cell death was detected 48 hours following the infection (Panel B). Data represent the mean (± SEM) of 6 independent data points.

TRAIL resistance in breast cancer cells remains to be tested yet. Quantitative Real Time RT-PCR assays were developed in order to assess the level of TRAIL receptor gene expression in breast carcinoma cells. While all TRAIL receptors were detectable in MCF7 breast carcinoma cell line, the level of TRAIL-R4 decoy receptor gene expression was the highest among the four (Figure 7, Panel A). This intriguing observation is consistent with a previous report suggesting that transient TRAIL-R4 overexpression protected target cells from TRAIL induced cytotoxicity [45]. TRAIL R4 is known to protect cells from apoptosis by acting both as a decoy receptor and an antiapoptotic signal provider. While Real Time PCR assay is useful in assessing the level of gene expression on mRNA levels, obviously this assay does not necessarily reflect TRAIL receptor composition on cell surface. For this reason, conventional flow cytometry analysis was carried out in order to determine the level of TRAIL receptor protein expression on cell surface. Despite the presence of TRAIL death receptors, substantial levels of TRAIL-R4 decoy receptor expression were detectable on the surface of MCF7 breast carcinoma cells (Figure 7, Panel B). On top of that, TRAIL sensitive MDA-MB-231 cell line (Figure 9) displayed very low levels of TRAIL-R4 decoy receptor expression on cell surface (Figure 8, Panel B). Neither of the cell lines expressed detectable levels of TRAIL-R3 decoy receptor on surface. Intriguingly, administration of a DcR2 siRNA approach lowered surface TRAIL-R4 expression and sensitized MCF7 breast cancer cells to TRAIL (Figure 10).

Conclusion

Our results demonstrated that the expression of TRAIL-R4 decoy receptor but not TRAIL-R3 appeared to correlate well with TRAIL resistance phenotype observed in MCF7 breast cancer cells. Further screening of another breast cancer cell line, MDA-MB-231, revealed that low levels of TRAIL-R4 expression on surface were correlated with TRAIL sensitivity. These results strengthen our argument that TRAIL-R4 but not TRAIL-R3 is the decoy receptor which appeared to influence TRAIL sensitivity in breast cancer cells. This is further confirmed by a DcR2 siRNA assay which suggested that down regulation of TRAIL-R4 expression sensitized MCF7 breast cancer cells to TRAIL. In addition, the inhibition of IKK pathway thereby NF-kB sensitized MCF7 cells to TRAIL induced apoptosis despite the expression of TRAIL-R4 decoy receptor on cell surface. Consequently, this complementary gene therapy approach involving IKK inhibition might be necessary to breakdown TRAIL resistance encountered in patients with breast cancer.

Abbreviations

TRAIL= Tumor Necrosis Factor (TNF)-Related Apoptosis-Inducing Ligand, EGFP= Enhanced Green Fluorescent Protein, MOI= Multiplicity of Infection, DcR2= Decoy receptor 2.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

ADS performed cell viability, Luciferase, Flow Cytometry, Real Time RT-PCR and siRNA assays, ED assisted ADS with adenovirus preparation, CA performed AdEGFP transduction assays, NE cultured breast cancer cells, SK optimized flow cytometry assays, SS participated in the coordination and execution of the study. All authors read and approved the final manuscript.

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