MODEL CHECKING OF APOPTOSIS SIGNALING PATHWAYS IN LUNG CANCERS

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ABSTRACT

MODEL CHECKING OF APOPTOSIS SIGNALING PATHWAYS IN LUNG CANCERS

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Model checking is a formal verification technique which is widely used in different areas for automated verification and analysis. In this study, we applied a Model Checking method to a biological system. Firstly we constructed a single-cell, Boolean network model for the signaling pathways of apoptosis (programmed cell death) in lung cancers by combining the intrinsic and extrinsic Apoptosis pathways, p53 signaling pathway and p53 - DAP Kinase pathway in Lung cancers. We translated this model to the NuSMV input language. Then we converted known experimental results to CTL properties and checked the conformance of our model with respect to biological experimental results. We examined the dynamics of the apoptosis in lung cancer using NuSMV symbolic model checker and identified the relationship between apoptosis and lung cancer. Finally we generalized the whole process by introducing translation rules and CTL property patterns for biological queries so that model checking any signaling pathway can be automated .

Keywords: Formal Verification, Model Checking, Signaling Pathways, Apoptosis

ÖZ

AKCİĞER KANSERİ VAKALARINDA APOPTOZ SİNYAL YOLLARININ MODEL DENETLEMESİ

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Model denetleme farklı alanlarda otomatik geçerleme ve analiz yapmak için yaygın olarak kullanılan bir formel geçerleme tekniğidir. Bu çalışmada bir model denetleme yöntemini biyolojik bir sisteme uyguladık. Öncelikle akciğer kanseri vakalarında apoptoz yani programlanmış hücre ölümü sinyal yollarını, boole değerler alabilen bir ağa dönüştürdükten sonra NuSMV modeline çevirdik. Bu ağı içsel ve dışsal apoptoz yolları ile p53 sinyal yolunu ve akciğer kanserlerinde gözlenen p53 - DAP Kinaz yolunu birleştirerek oluşturduk. Sonrasında oluşturduğumuz bu modeli, deneysel sonuçlara uygunluk açısından kontrol ettik ve akciğer kanserlerinde apoptoz dinamiklerini sembolik model denetleyici NuSMV kullanarak sorguladık ve apoptoz ile akciğer kanseri arasındaki ilişkiyi belirledik. Son olarak bütün süreci, çevrim kuralları ve biyolojik sorgular için zamansal özellik paternleri sunarak genelledik. Böylece herhangi bir sinyal yolunun model denetleme sürecinin otomatize edilebilmesine zemin hazırladık.

Anahtar Kelimeler: Formel doğrulama, Model Kontrolü, Sinyal Patikaları, Apoptoz

To My Family

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CHAPTER 1

INTRODUCTION

1.1. Motivation

Biological network models contain large amount of data which creates a need for model validation tools. Model validation requires comparison of predicted model with the experimental data by querying the model for some desired properties.

Some of the important types of systems network are signaling pathways and genetic regulatory networks. These are complex networks of interacting genes, proteins and molecules in order to control the functions of living organisms (Batt et al., 2005). In order to increase understanding of these networks in an organism, e.g. the cell response to a specific element or the behavior of a tumor which evades apoptotic death, mathematical tools are needed for modeling and simulation.

Fisher and Henzinger claim that formal verification approaches and techniques must be integrated into research area of biology in order to extend potential of executable biology as a mainstream technique (Fisher et al., 2007). 'Executable biology' is the approach of applying executable computer algorithms to the biological area for the construction of biological system models (Fisher et al., 2007). There are several computational models which can be used for analyzing biological networks such as Boolean networks, Petri nets, interacting state machines, process calculi and hybrid models. Each of these modeling approaches is applicable to different biological

processes. Petri nets and process calculi are best suitable for metabolic and signal transduction pathways. Interacting state machines and hybrid models are particularly suitable for intracellular signaling and cell-cell interactions. Boolean networks are applied to gene regulatory networks. Modeling offers great advantages for integrating and evaluating information and forming predictions which enables to focus experimental work. These system-level models will increase our knowledge about biological systems and eventually lead to the investigation of new therapies (Fisher et al., 2007).

It is important to understand the molecular events that contribute to drug-induced apoptosis, and how tumors evade apoptotic death. Defects in apoptosis are implicated in both tumorigenesis and drug resistance, and these defects are cause of chemotherapy failures. Lung cancer is a major cause of cancer deaths throughout the world (Shivapurkar et al., 2003). In the light of the recent studies we examined whether the relationship between apoptosis and lung cancer can be identified using modeling approaches and we investigated how to perform this analysis in a systematic way.

1.2. Problem Definition

The purpose of this study is to apply model checking technique to a biological system in order to investigate the relationship between lung cancer and apoptosis. In addition, we aimed to generalize the whole process which includes translation to NuSMV input language from signaling pathways and conversion of experimental data to Ctl properties in order to query whether the experimental data matches with the current model.

1.3. Limitations

The major limitation of this study is the state explosion problem. It is the time and memory overflow due to exponential growth of the state space with increasing number of variables. There is a huge amount of interconnected data about biological systems and we had to limit our scope in order to cope with the state space explosion problem.

Each query about model increases the execution time and it is not possible to generate all specifications for corresponding experimental results which is another limitation of our study.

1.4. Overview

The rest of the thesis is organized as follows. Chapter 2 details the concept of model checking, bioinformatics in terms of pathways. Previous research and applications on model checking of biological systems are also presented in Chapter 2. Information about lung cancer and apoptosis is given in Chapter 3 as background. Chapter 4 presents the methodology for constructing NuSMV model. Rules extracted and example NuSMV codes for each rule are presented. Chapter 5 is where the selected properties and their categories are proposed. The CTL properties, their results and comparison with the known experimental data are given in this chapter. Finally, Chapter 6 presents the conclusion of the thesis and future work directions.

CHAPTER 2

LITERATURE REVIEW

The background and literature review are provided in this Chapter. The background section introduces main subjects: Bioinformatics, pathways and model checking. Meanwhile the related work section introduces research that has been done and that is being performed in the area of model checking in bioinformatics domain.

2.1. Background

2.1.1. Bioinformatics

Systems biology is an interdisciplinary field, which is the combination of biology, chemistry, physics, mathematics, electrical engineering and computer science. Main purpose of this field is the integration of data about genes and proteins and investigation of how these elements function in a biological system (Fisher et al., 2007).

The goal of systems biology is to help biologists to increase predictive manner in the study of generation, diversification and maintenance of biological processes by the use of mathematical models which have a proven track record in other physical and engineering sciences.

Over the past few decades systems biology emerged by the completion of various genome projects. The large increase in data coming from these projects and the rapid developments in other molecular research technologies have produced a tremendous

amount of information which exceeds the human capacity to analyze it (Fisher et al., 2007). Bioinformatics is the name given to mathematical and computing approaches used to understand this huge amount of information and biological processes.

In other words bioinformatics is the application of computer science and information technology to the field of biology. The primary objective of bioinformatics is to increase understanding of biological mechanisms. Different from other approaches it focuses on developing and applying computationally intensive techniques such as pattern recognition, data mining, machine learning algorithms and visualization to achieve its objective. Drug design, drug discovery, protein structure alignment, prediction of gene expression, protein-protein interactions are the major research efforts in the field.

Today bioinformatics includes the creation and advancement of databases, algorithms, computational and statistical techniques and theories to solve formal and practical problems arising from the management and analysis of biological data.

Common activities in bioinformatics include mapping and analyzing DNA and protein sequences, aligning different DNA and protein sequences to compare them and creating models of protein structures and intracellular and extracellular processes and interactions. Intracellular and extracellular processes are generally shown by different types of networks or pathways such as metabolic pathways and signaling pathways which are defined in the following section.

2.1.2. Types of biochemical networks

A cellular system can be viewed from different perspectives such as in the level of molecules, interactions, networks, signaling pathways in subnetworks and eventually global networks (Zhang, 2009). Biomolecular networks allow visualizing and describing of intracellular molecular interactions of cellular system by using available metabolic and gene regulation experimental data (Zhang, 2009), as well as representation of many biological processes such as metabolism, gene regulation, signal transduction. Interactions of a biomolecular network consist several type of

pairwise interactions between genes, proteins, enzymes and molecules (Zhang, 2009). Pathways are subsets of networks. Some important pathway types are defined below.

2.1.2.1. Metabolic Pathway

Metabolic pathways are series of chemical reactions and their occurrence within a cell changes the state of a principal metabolite. Enzymes, vitamins, minerals are required for the cataliziation and proper functioning of these reactions. Due to involvement of many chemicals, metabolic pathways are often quite complex. In addition, different pathways exist within a cell. The cross-talk of these pathways is called the metabolic network. Pathways maintain the stability and regulation of the internal environment of an organism. Creation of new molecules is the final product of Anabolic (synthesis) and Catabolic (break-down) pathways which often work in collaboration with each other.

An initial molecule is modified step-by-step in a metabolic pathway. The resulting product can be:

- used immediately as end-product
- used to start another pathway
- stored within the cell for future usage.

2.1.2.2. Signaling Pathway

Signal transduction is the process by which an extracellular signaling molecule activates a membrane receptor that in turn alters intracellular molecules creating a response (Silverthorn, 2007). When a signaling molecule activates a certain receptor on the cell membrane, it causes a second messenger to continue the signal into the cell and a physiological response is derived. Signaling pathways are the series of these reactions which performs the signal transduction.

2.1.3. KEGG

In our work, intrinsic and extrinsic pathways of Apoptosis are obtained from **KEGG** (**Kyoto Encyclopedia of Genes and Genomes**) database. It is a collection of online

databases containing chemicals, genes and several types of pathways[1]. Molecular interaction networks in the cells and their specific variants for particular organisms are recorded in this database.

The KEGG database is a part of the systems biology approach which is used for the easy retrieval of data to model and simulate biological processes.

KEGG contains Pathway Database for molecular interaction networks; the gene database for the information about genes and proteins comes from genome projects, compound and reaction databases for biochemical compounds and reactions.

2.1.4. Modeling and types of models

The construction of models for biological systems is the core of systems biology (Fisher et al., 2007). The main goal of modeling is to supplement researchers understanding of biological system properties (local, global) and corresponding behaviors. We briefly characterize two types of computational models according to their goals: kinetic (dynamic) and structural models:

2.1.4.1. Kinetic Models

Ordinary differential equations and stochastic processes are common modeling techniques for cellular systems. In order to obtain more quantitative information about functions and mechanisms of cellular systems, dynamic simulations can be utilized (Zhang, 2009). Researchers can test their understanding by using simulation results and explore "what-if" scenarios to make predictions about the parts of the system which have not yet been studied. Results can be used to design new biological systems.

2.1.4.2. Structural (Topological) Models

Another class of methods in Systems Biology is parameter-fee analysis of cellular networks which is called structural or topological analysis (Klamt et al, 2007). Structural or topological models are represented in graphical form. These graphs can

[1] Retrieved August 18, 2011, from http://www.genome.jp/kegg/pathway.html

be directed, undirected or combination of these and consist of nodes and edges. "Nodes represent genes, gene products, proteins, chemical compounds or small molecules and edges represent various types of interactions or associations between pair of nodes, e.g. metabolic events, protein/protein-nucleotide interactions, regulatory relationships or signaling pathways" (Zhenjun Hu et al, 2007).

Both of these analyses are useful for deeper understanding of biological systems in different ways. Structural model analysis enhances the knowledge about network-wide interactions and causal relations but can-not provide quantitative answers. Meanwhile dynamic analyses which are performed on kinetic models can reveal quantitative dynamic properties but the dynamic behavior is often based on the network structure (Klamt et al, 2006).

Hence, we first focused on the analysis of structural (topological) model of our signaling pathways in our study. Dynamic analysis can be performed upon this study.

2.1.5. Boolean Network

Boolean network (BN) modeling approach is used in our study, for depicting the signaling pathway. A BN is an abstraction of a dynamic system, and has been previously applied to gene regulatory network and signaling pathway studies (Gong et al., 2011). A Boolean network a directed graph with set of nodes and a Boolean transfer function for each node. The state of each node in a BN can be either Active(1) or Not Active(0) at any time step, except for the nodes which correspond to the external (control) signal. The Boolean transfer function describes the transformation of the state of each node for the next time step.

The following example illustrates how a Boolean network can model a signaling pathway together with its end products (the outputs) and the substances from the environment that affect it (the inputs).

1. Each metabolite and state is represented by a node.
2. Each connection (edge) in the graph represents physical association such as activation or inhibition of the target metabolite.

3. Each node in the graph can be “active” or “not active” states.
4. Time proceeds as discrete steps. At each step, the new state of the principal node is determined by the states of nodes which activates or inhibits it.

Comparing simulation results with time series observations can be used to test the validity of the model. Given a BN model, one of the systems biologist’s interests is to verify sequences of signal transduction which will drive the network to a pre-specified state at or before a pre-specified time. Model checking technique can be used to solve this problem.

2.1.6. Model Checking

Ensuring the correctness of biological systems becomes increasingly important as the complexity of these systems grow. Building models is the first step of high level assurance but checking these models with effective analysis techniques is also very important. Simulation is the most widely used technique in industry which is useful for finding bugs and errors i.e. proving the system's incorrectness, but not sufficient to prove the system's correctness.

There is another family of verification techniques that are characterized as formal methods, which uses mathematical techniques in order to prove the system correctness. Theorem Proving and Model checking are types of formal methods:

- **Theorem Proving:** the system is specified in a logical system and deductive verification techniques are applied to prove properties. This approach is mostly manual.
- **Model Checking:** instead of manual deduction technique model checking uses exhaustive state space exploration in order to prove properties.

In this thesis we are mainly concerned with the model checking technique. As shown in Figure 2.1, an automated tool called *model checker* is used to check a model M against its expected property P, i.e. to prove or disprove the formula M |- P. The outcome is either a correctness confirmation that shows the property P holds for

model M, or a counter example which shows the program trace that violates the property P.

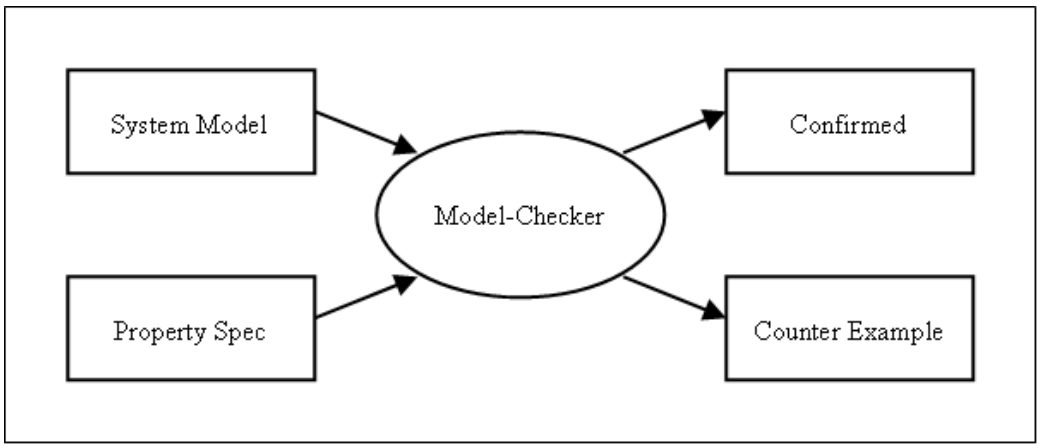

Figure 2.1: The model-checking process

Typically the system model M is specified in some type of state machine notation, and the model checker explores the state-space of M to determine the validity of desired property P.

The main advantage of Model checking technique against other formal verification techniques is its ability to perform automatic verification and generation of counterexamples. The use of model checking requires modeling the system and determining the properties which will be verified. Once these steps are done, the verification of these properties for the model is a push button process.

Model checking algorithms operate on a model named Kripke structure. Kripke structure is a finite state machine where each state has a label. These labels show the Boolean expressions defined in the system which evaluates to true for that state. These expressions are called as atomic properties. Although algorithms are defined in Kripke structure, modeling is performed with the meta languages which model checking tools are accepted as input languages in practice. For example if model checking tool is SPIN (Holzmann, 1997), the system is modeled with Promela language. The main challenge during modeling is the exponential growing size of state space and the state space explosion problem. In order to shrink the state space, a

model is formed by applying abstraction techniques. Abstraction makes model checking feasible but it should be carefully handled in order not to mislead verification results.

The properties which will be verified for the defined system are expressed with Temporal Logic. Temporal logic is used to define propositions whose correctness is dependent to time. Linear Temporal Logic (LTL) and Computation Tree Logic (CTL) are the most widely used property specification logics in model checking. In LTL, the model of time is a sequence whereas in CTL, the model of time is a tree. In CTL quantification is performed over this computation tree.

The last step of model checking is to control whether the model satisfies the given properties. This step is performed automatically by the model checking tools. In case the model does not satisfy the given properties tools reports an execution path where the error is generated. Hence not only the existence or nonexistence of errors is shown but also how the error can be re-generated is explained.

2.1.7. CTL Semantics

There are four basic temporal operators (Clarke et al., 1999):

Invariant *p*	:	G *p*	(aka	*p*)	(Globally)
Eventually *p*	:	F *p*	(aka	*p*)	(Future)
Next *p*	:	X *p*	(aka	*p*)	(neXt)
p **Until** *q*	:	*p* U *q*			

Eight temporal operators exist in CTL: **AX, EX, AG, EG, AF, EF, AU, EU** where path quantifier A means "*for all paths*" and E means "*there exist a path*". We used these operators to check generic rules such as "something is never activated in all paths" or "some event always happens after something is deactivated".

The following is the semantics of CTL properties (Clarke et al. 1999)
Given a **state** s and CTL properties **p** and **q:**

$s \models p$	iff	$p \in L(s)$ where $p \in AP$
$s \models \neg p$	iff	not $s \models p$
$s \models p \wedge q$	iff	$s \models p$ and $s \models q$
$s \models p \vee q$	iff	$s \models p$ or $s \models q$
$s0 \models EX\ p$	iff	there exists a path s0, s1, s2, ... such that $s1 \models p$
$s0 \models AX\ p$	iff	for all paths s0, s1, s2, ..., $s1 \models p$
$s0 \models EG\ p$	iff	there exists a path s0, s1, s2, ... such that for all $i \geq 0$, $si \models p$
$s0 \models AG\ p$	iff	for all paths s0, s1, s2, ..., for all $i \geq 0$, $si \models p$
$s0 \models E(pUq)$	iff	there exists a path $s_0, s_1, s_2, ...$, such that, there exists an $i \geq 0$ such that $s_i \models q$ and for all $0 \leq j < i$, $s_j \models p$
$s_0 \models p\ AU\ q$	iff	for all paths $s_0, s_1, s_2, ...$, there exists and $i \geq 0$ such that $s_i \models q$ and for all $0 \leq j < i$, $s_j \models p$

If we express these eight operators in plain English:

AX p means eventually p will become true in the next step.
EX p means it is possible that p will become true in the next step.

AG p means eventually p is true all the time.
EG p means it is possible that p is true at s0 and stays true from then on.

AF p means eventually p will become true in the future.
EF p means it is possible that p will become true in the future.

A (p U q) means a state satisfying q is **necessarily** preceded all the time by a state p
E (p U q) means a state satisfying q is **possibly** preceded all the time by a state p

2.1.8. NuSMV

NuSMV (Cimatti et al., 2002) is a model checker which uses SMV modeling language. Temporal properties are specified by using CTL. NuSMV is used in this

thesis since the model to be checked is close to synchronous execution model. The tools like SPIN are mostly suitable for asynchronous execution models. The first application area of SMV modeling language is hardware verification (McMillan, 1992). This reflects the structure of the SMV language such that the complex data structures and function definitions are not supported directly. However Boolean and enumeration types are supported in a rich way.

2.1.9. Modeling Language

In this section basic SMV structures used while modeling is explained generally. Basically an SMV model is a definition of finite state machine. The main machine is defined as Module main and it starts the execution. SMV model can be divided into different sub modules and variables are defined in each sub module in the same way. Semantically these sub modules execute in parallel.

VAR p1: process proc1();

Modules can take parameters. Each module composed of VAR and ASSIGN sections. In VAR section Boolean or Enumerated variables or bounded integer variables are defined which constitutes the state space. For example:

VAR b: boolean; VAR e: {e1, e2}; VAR i: -5..5;

In ASSIGN section values are assigned to defined variables by using init() and next() expressions. init() expression is used to initialize the variables. next() expression is used to assign the rules set which determine the value of the variable in the next state. For example, the below expression shows the state transition for the variables b, e and i declared before. According to this rule, variable b is reversed and the value of variable e is set to e1 and value of the variable i is set to 0 in the next time step (Philipps et al., 1999):

next(b) = !b & next(e) = e1 & next(i) = 0

Case expression is used for larger rule definitions.

```
case
  c1: e1;
  c2: e2;
  ...
  cn: en;
esac;
```

This expression is same with the following (Philipps et al., 1999):

```
(c1 -> e1) & (!c1 & c2 -> e2) &
... (!c1 & !... & cn -> en)
```

If more than one rule is satisfied, the condition which will be executed is non-deterministic. Non-determinism is a part of abstraction. Generally there is a default case and in SMV language this case is expressed by using true (1) condition which will always be satisfied. It means "if any of the conditions are satisfied this condition will be executed".

2.2. Related Work

There have been several studies of model checking for cell biology. Gong et al. (2011) applied model checking to the study of a biological system -the HMGB1 Boolean network.

They claim that the Boolean network modeling and Model Checking provide an alternative way and new insights into the study of the HMGB1 signaling pathway in pancreatic cancer. In light of that study, we examined whether the model checking can be effectively applied to Apoptosis Pathways in Lung Cancer. Also we have generalized the rules applied while transferring the pathway model into the NuSMV model and the temporal logic patterns that we used to control our model. 33 variables are included in their study which means the modeled system is composed of 33 metabolites or proteins. Whereas in our study, 58 variables are modeled i.e. almost

two times more than their variables are used which increases the state space nearly four times.

Batt et al. (2005) propose an approach towards model validation in order to address two main challenges. First of all, the precision of the model predictions and the experimental data need to be brought in agreement. The second challenge is to ensure that the comparison of model predictions with experimental data is efficient and reliable. Their approach is the qualitative modeling and simulation of genetic regulatory networks and they supported their process with a computer tool named Genetic Network Analyzer (GNA).

They use piecewise-linear differential equations in order to simulate the network dynamics. Instead of numerical values, inequality constraints extracted from experimental data are used by the method. This method can be suitable for simulation purposes if the researcher is interested in the system behavior in terms of the quantity of materials in the system. However, it is not suitable for asking qualitative queries related with reachability, consequence and sequence of pathways which will be explained in this thesis in the following chapters.

Fisher and Henzinger (2007) call the approach of constructing computational models of biological systems as 'executable biology'. Figure 2.2 shows the methodology of executable biology (Fisher et al., 2007).

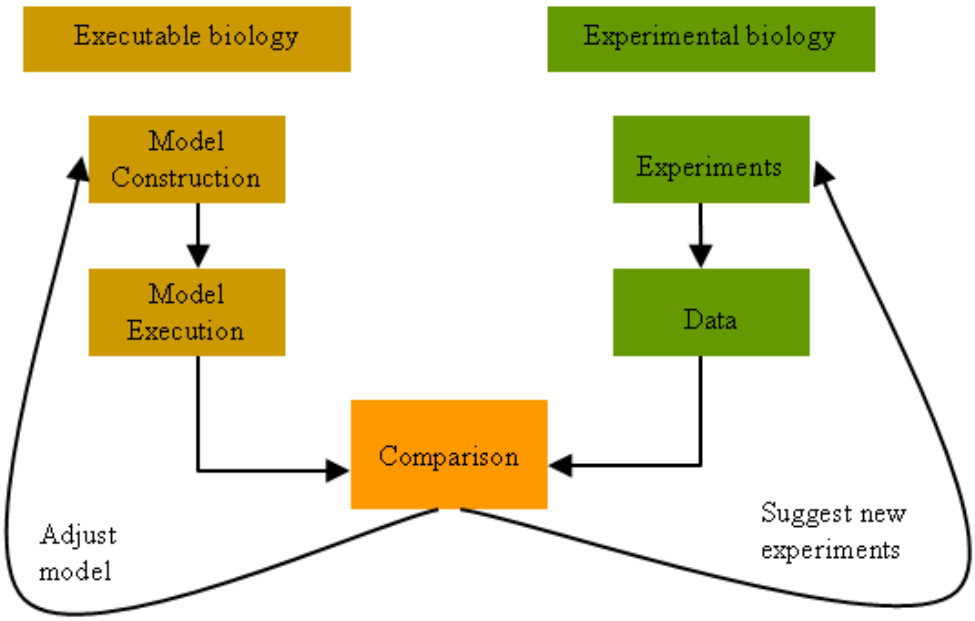

Figure 2.2: The methodology of executable biology.[2]

In their review, the applicability and benefits of modeling biological systems and the challenges in integrating biology and computer science is surveyed. In their paper, comparison of Executable Biology modeling approaches are presented such as Boolean networks, Petri nets, interacting state machines, process calculi and hybrid models as shown in Table 2-1.

Table 2-1: Comparison of Executable Biology modeling (Fisher et al., 2007).

Modeling Approach	**Boolean Networks**	**Petri Nets**	**Process Calculi**	**Interacting State Machines**	**Hybrid Models**
Referenced applications	Gene regulatory networks	Metabolic and signal transduction pathways	Metabolic and signal transduction pathways	Intracellular signaling, cell-cell interactions	Cell-cell interactions
Examples of modeled systems	Yeast cell-cycle regulation	EGFR signaling pathway, tryyophan regulatory network, glycolysis pathway	RTK-MAPK and FGF signaling pathways	T-cell activation, thymo-cytes differentiation, *C. elegans* vulval development	Delta-Notch decision, bacteria quorum sensing

[2] Fisher et al., 2007

Table 2-1 (cont.)

Modeling Approach	Boolean Networks	Petri Nets	Process Calculi	Interacting State Machines	Hybrid Models
Examples of description languages	-	-	Pi calculus, Ambient calculus, Brane calculus	Statecharts, Reactive Modules	Hybrid automata
Time	Discrete	Discrete	Continuous	Discrete	Continuous
Concurrency	-	Synchronous and asynch.	Synchronous and asynch, and stochastic	Synchronous and asynch.	Synchronous
Structuring	-	-	Compositional	Hierarchical and compositional	Compositional
Referenced analyses	Reasoning about stability and robustness	Static analysis of system dynamics	Dynamic analysis (simulation) of molecule quantities	Static analysis of system dynamics (model checking)	Reasoning about stability and system dynamics
Examples of software tools	Matlab	Pathalyzer	BioSPI, SPiM, PEPA	Rhapsody, Mocha	Matlab, Charon

Each of these modeling approaches is suitable for different biological processes. According to referenced applications, Boolean networks are applied to gene regulatory networks. For metabolic and signaling pathways, Petrinets are used for discrete time and process calculi are used for continuous time. In our work we modeled Apoptosis signaling pathway in Lung Cancer by using Boolean Network modeling approach since it is the simplest structural modeling which is sufficient to define interactions in pathways used in our model.

Efroni et al. (2003) used the language of state charts, which is a visual language for specification and modeling. In this work, events in biological structure of thymus, e.g thymocyte movement, are modeled in a three-dimensional representation. They utilized state charts from different levels and showed their interrelationships with this representation.

The end result of their study is a running simulation. The cells and molecules are animated like interactive movies. This interactivity allows manipulation and representation of the data that generated the simulation and the data that is generated by the simulation. But the main concern of this thesis is to verify the more generic hypothesis which can be inferred from the model rather than simulation. Interactions

can be observed by simulation such as which items are activated or deactivated if we increase a specific item. However we want to check more generic rules such as "something is never activated in all paths" or "some event always happens after something is deactivated".

The modeling of biomolecular networks work of Chabrier et al. (2003) is closely related to our approach. They have applied symbolic model checking techniques to the querying and validation of both quantitative and qualitative models of biomolecular systems. The categorization of the CTL queries in the mammalian cell cycle control model is very similar with queries that are used in our work. However their modeling process is not explained in that paper but we generalize not only CTL queries but also our modeling process. They claim that their experiments show some advantages over simulation.

They have also shown that constraint-based model checking can be applied in quantitative models such as gene interaction described by differential equations. This approach can be helpful for future work of our apoptosis and cancer relation study. If we can obtain more quantitative data we may apply stochastic model checking methods for further information.

Another work upon statistical model checking is the automated analysis of T-Cell receptor signaling pathway by Clarke et al. (2008). They present an algorithm, called BIOLAB, for formally verifying properties of stochastic models of biochemical processes.

Although statistical model checking adds value to model verification we wanted to focus qualitative data instead of quantitative data. We used pathway data which is not quantitative and can be expressed with Boolean Networks since the differential models are not provided. Quantitative analysis may be concerned for future direction of our study for further analysis of our model.

CHAPTER 3

PROBLEM DOMAIN

The problem domain is provided in this Chapter which introduces the following main subjects: Apoptosis (programmed cell death) and Lung Cancer relation, intrinsic and extrinsic pathways of Apoptosis and the major factors observed in Lung cancer studies which effect Apoptosis. Beside the signaling pathways of apoptosis, p53 signaling pathway and a part of p53 - DAP Kinase pathway in Lung cancers are used in our study for constructing a single-cell, Boolean network. After the explanations of these pathways, the diagrams taken from KEGG database are shown at the end of this chapter.

3.1. Cancer and Apoptosis

"Apoptosis is an evolutionarily conserved and genetically regulated form of cell suicide which plays an important role in development and in the maintenance of tissue homeostasis in multicellular organisms" (Webb et al., 1997; Wyllie, 1997). In complex organisms like human, cellular proliferation is a need for maintenance, repair and growth. However if the genes which regulate the cell proliferation mutate due to several factors, the cancer threat arises as a result of uncontrolled growth. The cell must protect the balance between death and growth. "Thus, the organism must find a way to allow cellular proliferation only when needed while effectively suppressing this activity at other times" (Shivapurkar et al., 2003). Cellular proliferation and apoptosis mechanisms are coupled in order to achieve the above balance.

There are different triggers which activates cell apoptosis. DNA damage, stress signals, and hypoxia are examples of external factors whereas death ligands such as FasL, TNF and TRAIL are internal initiators (Prendergast, 1999). Some of these stimuli are also observed in the incipient tumors. It is claimed that triggers of cell growth and mutation, also induce apoptotic stimuli and if these are not inhibited affected cells are removed with apoptosis automatically (Shivapurkar et al., 2003).

Mutated or transformed cells are eliminated from the body as a result of Apoptosis. However, tumor cells and incipience of them have a strong resistance to apoptosis usually in multiple levels. Hence, cancer cells can evade apoptosis which is their major hallmark (Shivapurkar et al., 2003). There is an interesting fact about cancer tissues that is the increased rate of both apoptosis and resistance to it. the reason of this anomaly is the enormous pressure on the affected cells to go to death and their resistance in response to be able to survive (Shivapurkar et al., 2003). Apoptosis is a highly complex interacting network which includes more than 150 genes and it is redundant to many signaling pathways (Shivapurkar et al., 2003).

There is a family of caspases which orchestrates programmable cell death. It is known that 14 mammalian caspases regulate the apoptosis. These can be categorized as initiator and effector caspase. Initiator or upstream caspases includes CASP8, CASP9, and CASP10. These are stimulated by proapoptic triggers and activate the effector or downstream caspases such as CASP3, CASP6, and CASP7.
As shown in Figure 3.1 there are two major pathways for apoptosis. One of them is initiated by CASP8 and the other is by CASP9 (Shivapurkar et al., 2003). These pathways are named as Extrinsic pathway and Intrinsic pathway respectively. The Extrinsic pathway is initiated by death receptors and the Intrinsic pathway is activated by mitochondrial stimuli.

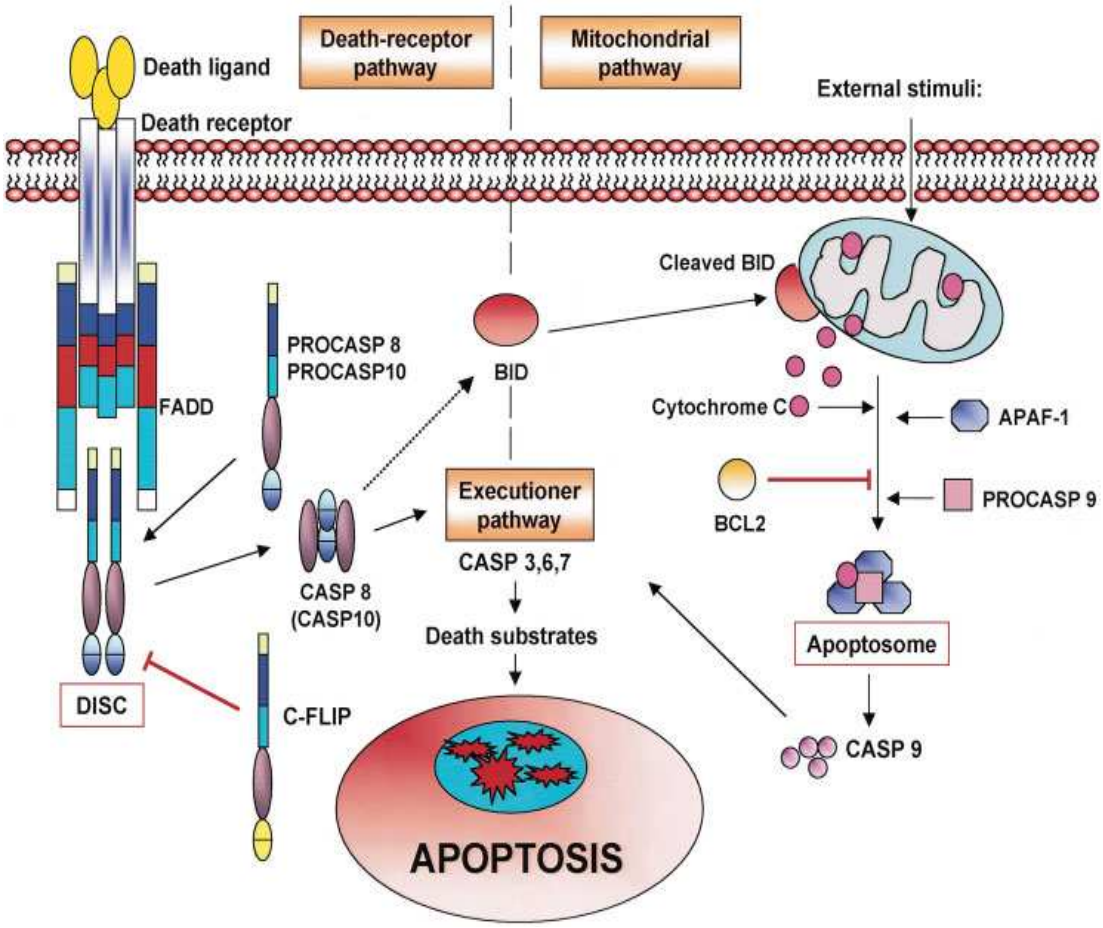

Figure 3.1: The major pathways to Apoptosis[3]

3.1.1. Extrinsic Pathway

Activation of caspases is started by the death inducing ligands such as FasL TNF, or TRAIL. FAS is the receptor for the death ligand FasL, TNFR1 is for TNF, and DR4 and DR5 are for TRAIL respectively. Their binding triggers apoptosis via adaptor proteins such as TRADD-FADD compound. Then procaspase 8 is recruited.

Either CASP8 or (tBid) is activated which mediates the cytochrome c release from mitochondria. In the study of Shivapurkar et al. (2003), it is proved that both CASP8 and CASP10 caspases may have an essential role in the initiation of apoptosis. Due to this information we queried the relation of CASP8 and Apoptosis in our properties defined in Chapter 5. According to this review all lung cancer lines expressed

[3] Shivapurkar et al., 2003

CASP10 and CASP3. Hence CASP10 and CASP3 are defined as the control nodes of our model.

3.1.2. Intrinsic Pathway

The other initiator pathway of apoptosis is the release of cytochrome c in response to several internal stimuli such as stress signals, hypoxia etc. Cytochrome c binds to and activates the adaptor protein Apaf-1. Then procaspase 9 and Apaf-1 creates a multiprotein complex. After recruitment of this complex Procaspase 9 is started which initiates the activation of downstream caspases. However, this sequence of caspase activation can be interrupted by a family of inhibitor proteins called IAPs. These proteins bind to the active caspases and inhibit them. IAPs can be inhibited also by Endonuclease G or Apoptosis-inducing factor (AIF) proteins which are included in apoptosis associated with DNA-fragmentation (Shivapurkar et al., 2003).

3.2. Lung Cancer and Apoptosis

"Lung cancer is the leading cause of cancer deaths in the world with over one million cases diagnosed every year" (Shivapurkar et al., 2003; Parkin et al., 2001). Human lung cancers are categorized into two major types, such as small cell lung cancer (SCLC) and non-small cell lung carcinoma (NSCLC) (Shivapurkar et al., 2003; Travis et al., 1995). Figure 3.2 shows the apoptotic index relation with different cancer types (Shivapurkar et al., 2003). Although there are several differences between SCLC and NSCLC types, we focused on the common properties which are applicable to both of these types in our study.

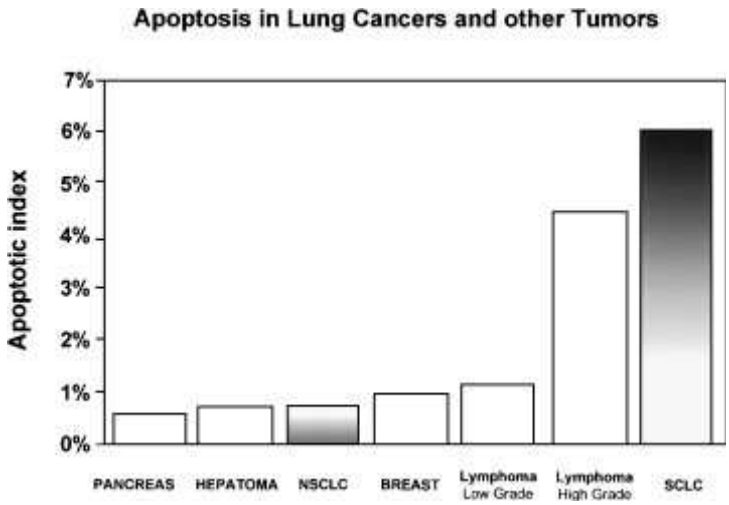

Figure 3.2: Apoptosis in Lung Cancers and other Tumors[4]

According to Fine et al. (2000), the knowledge about lung cancer tissues is immature relative to other major cancers due to fewer articles on apoptosis in lung cancers. Although recent reports investigate the fundamental role of apoptosis in lung cancer, there is still lot more to learn about this highly complex network which contains nearly 150 genes (Shivapurkar et al., 2003).

Joseph et al. (1999) revealed that overexpression of Bcl-2 and loss of caspases 1, 4, 8, and 10 is observed in lung cancer cells.

Another major finding which has been reported in recent studies is the overexpression of Bcl-2 and p53 proteins in lung cancer tissues (Brambilla et al., 1996; Chen et al., 1999; Kalomenidis et al., 2001; Sartorius & Krammer, 2002). We used this kind of information in our properties in order check our model. The role of Bcl-2 and p53 is explained in detail in the following sections.

3.2.1. Pro-apoptotic and Antiapoptic Bcl-2 Proteins

Mammalian Bcl-2 family proteins regulate the intrinsic pathway of apoptosis mainly by controlling the release of Cyctochrome c and other intermembrane mithocondrial proteins into the cytosol. It is found that Bcl-2 activation prevents apoptosis and

[4] Shivapurkar et al., 2003

contributes to proliferation of cancer cells which eventually leads to cancer development (Shivapurkar et al., 2003; Brambilla et al., 1996; Chen et al., 1999).

Some Bcl-2 proteins are pro-apoptic and promote apoptosis by enhancing the release, whereas others are anti-apoptic and inhibit apoptosis by blocking the release. Bax and Bak are the pro-apoptic Bcl-2 proteins. In mammalian cells, one of these proteins is required for intrinsic pathway of apoptosis. Bax is located in the cytosol and translocates to the mitochondria only after an apoptotic signal activates it. Anti-apoptotic Bcl-2 proteins such as Bcl-2 itself and Bcl-XL are located on the cytosolic surface of the outer mitochondrial membrane, the endoplasmic reticulum and the nuclear envelope. These proteins inhibit apoptosis mainly by binding to and inhibiting some pro-apoptotic proteins.

In lung cancers, an inverse correlation exists between the ratios of Bax and Bcl-2 expressions with respect to the grade of tumors. Bax expression decreases and Bcl-2 expression increases while the grade of tumor getting high (Brambilla et al., 1996). Since Bax is a pro-apoptotic member of the Bcl-2 family, this ratio can be thought as a measure of tumor resistance to apoptosis.

Sartorius and Krammer (2002), suggest the linkage of response to therapies to Bcl-2 family proteins for lung tumors. As shown in Figure 3.3 which is extracted from Joseph et al. (2000) Bcl-2 upregulation is observed in several types of Lung cancers hence we determined properties related with Bcl-2 in our study.

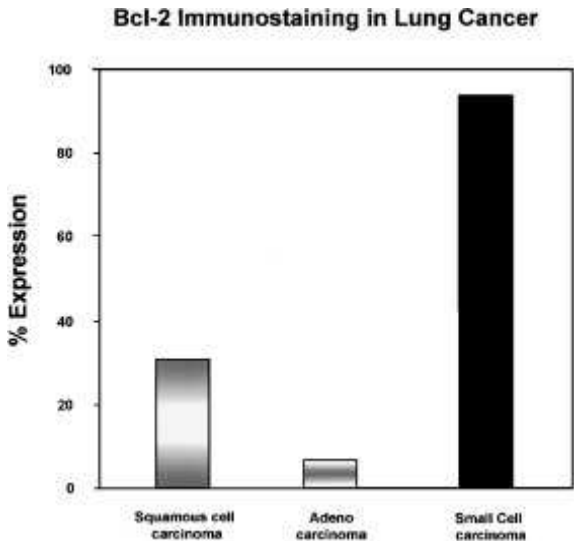

Figure 3.3: Bcl-2 regulation in different types of lung cancers.[5]

3.2.2. Inhibitors of Apoptosis (IAPS)

IAPs prevent cell death by inhibiting the activity of the initiator and effector caspases mainly caspase3 and caspase7. The transcription factor NF-kB is also inhibited by IAPs (Tang et al., 2006). NF-kB and anti apoptotic Bcl-2 proteins regulate the intrinsic pathway. The IAPs, inhibit all of the apoptotic pathways by inhibiting the executioner pathway. Considerable over expression of IAP has been reported in several tumor types including lung (Shivapurkar et al., 2003; LaCasse et al., 1998; Ferreira et al., 2001). This is the reason why we determined IAP as one of our control node.

3.2.3. Central Role of P53

The p53 has an essential role in apoptosis and used to prevent cancer formation. Its loss of expression is observed frequently in tumors causing inhibition of apoptosis. Activation of p53 may invoke many responses such as, DNA repair, cell cycle arrest, DNA fragmentation or apoptosis. It is a tumor suppressor gene, and its inactivation is observed in about 50% of human cancers (Soussi, 1996). It is known that p53 has several functions such as activation of Bax which is a pro-apoptotic Bcl-2 member, repression of antiapoptotic members, and activation of several genes including Apaf-

[5] Joseph et al., 2000

1, PTEN, and death inducing ligands' receptors. Shivapurkar et al. explains the role of p53 not only as the ‘‘guardian of the genome’’ but also as the ‘‘master regulator’’ of apoptosis. Due to its importance in lung cancers, many studies have been performed upon p53 variations in lung cancers. In about 50% of NSCLC and more than 70% of SCLC, p53 gene is mutated. Hence we selected properties related with p53 occurrence.

The Figure 3.4 shows the signaling pathway of p53- DAP kinase in Lung Cancer. We integrated this pathway into the Apoptosis pathway taken from KEGG database. According to this pathway, apoptosis will be degraded when DAP kinase, p53, p14/ARF or Bax is down regulated, or when the Bcl-2 is up regulated.

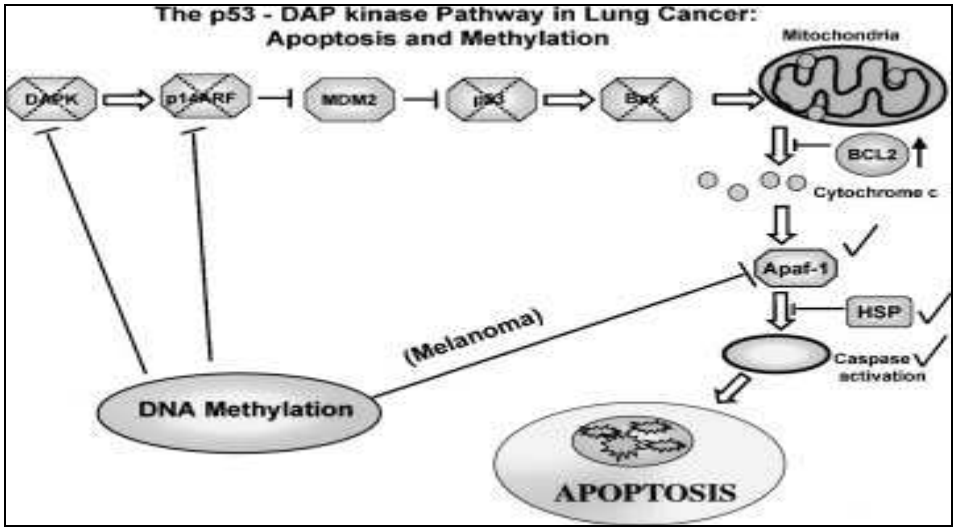

Figure 3.4: Disruption of the intrinsic (mitochondrial) pathway in lung cancers.[6]

3.2.4. Apoptosis Model from KEGG Pathway

Figure 3.6 which is taken from the KEGG database is the model we used in our study. It shows the intrinsic and extrinsic pathways of Apoptosis in detail relative to Figure 3.1. There are two alternative but overlapping major pathways to apoptosis. The extrinsic pathway is invoked by the death receptors. On the other hand the intrinsic pathway is activated by release of mitochondrial proteins. Then, a common executioner pathway is activated in both pathways. CASP9 is activated for the

[6] Shivapurkar et al., 2003

intrinsic pathway and CASP8 and probably CASP 10 are for the extrinsic pathway. When the initiator caspases are activated, they cleave and activate downstream executioner caspases which leads to cell death. Bcl-2 family member Bid is the cross-talk of these two pathways.

Due to the central role of p53 in apoptotic death, p53 signaling pathway which is shown in Figure 3.7 is also included in our Boolean network model. As a result, we constructed our cancer cell model as the combination of Apoptosis signaling pathways (shown in Figure 3.6), p53 signaling pathway (shown in Figure 3.7) and p53- DAP kinase pathway in Lung Cancer (shown in Figure 3.4).

The pathway is drawn and updated with the notation shown in Figure 3.5. Although there several protein-protein interactions, we only used two basic types in our study: activation and inhibition. Phosphorilation, dephosphorilation and indirect effect are considered as activation and dissociation is considered as inhibition. Complexes are defined in the same way with the chemical compounds.

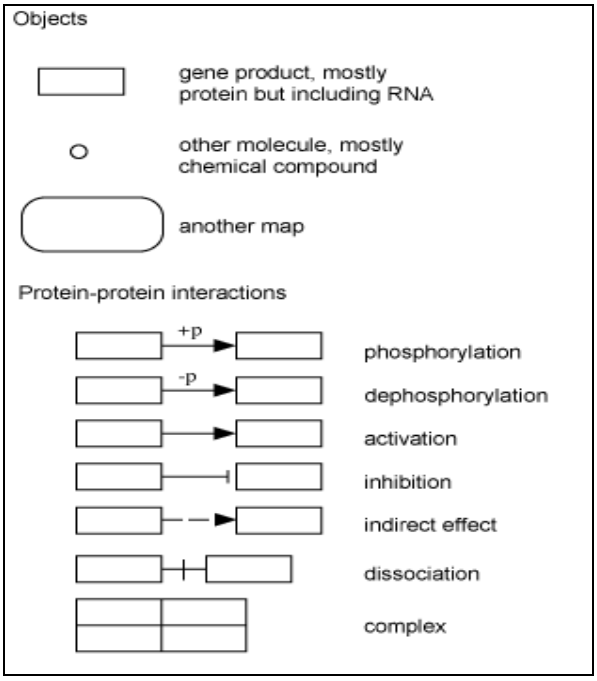

Figure 3.5: KEGG Pathway notations[7]

[7] Retrieved August 18, 2011, from http://www.genome.jp/kegg/pathway.html

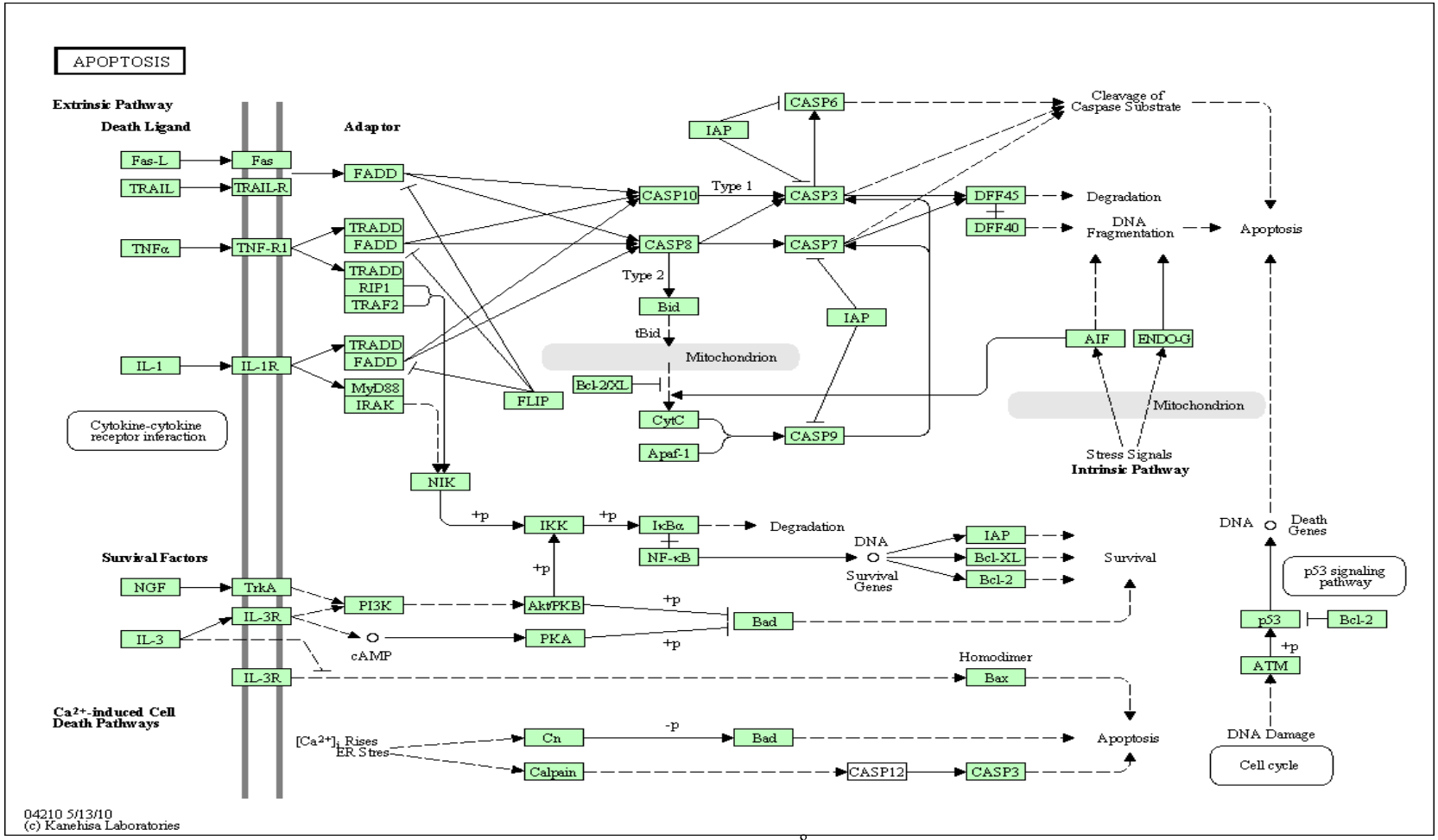

Figure 3.6: Apoptosis Process[8]

[8] Retrieved August 18, 2011, from http://www.genome.jp/kegg/pathway.html

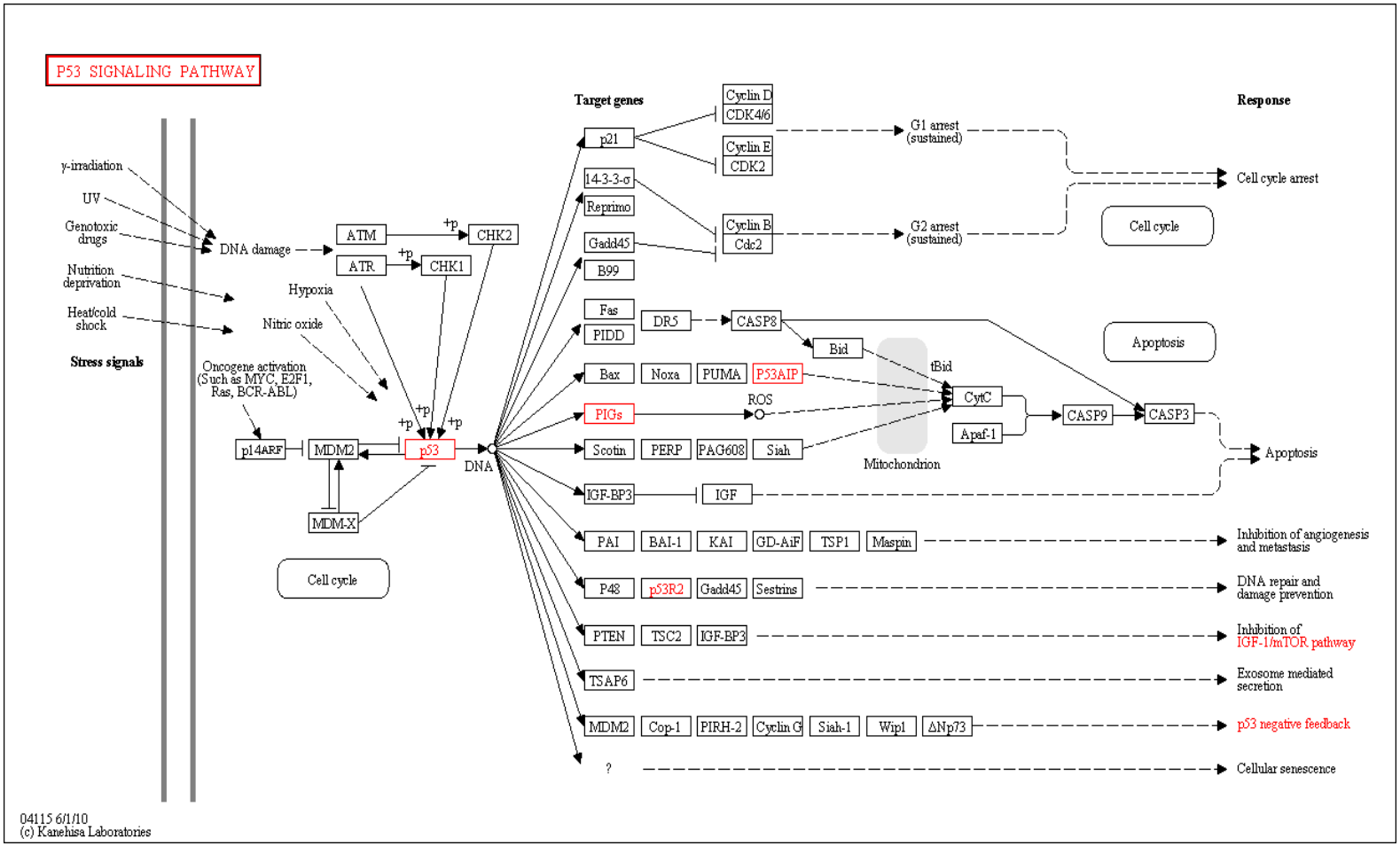

Figure 3.7: P53 Signaling Pathway[9]

[9] Retrieved August 18, 2011, from http://www.genome.jp/kegg/pathway.html

CHAPTER 4

METHODOLOGY

Chapter 4 presents the methodology for constructing NuSMV model using Boolean Network Modeling approach. Extracted rules for translation and example NuSMV codes for each rule are presented.

4.1. Boolean Network Model

Boolean Network Model is the intermediate transition between the NuSMV model and graphical representation of signaling pathways taken from KEGG database. We present the schematic view of intrinsic and extrinsic signaling pathways of Apoptosis in Figure 3.6, p53 signaling pathway in Figure 3.7, and p53- DAP kinase pathway in Lung Cancer in Figure 3.4 which are the raw formats for the Boolean Network model. Although there are several types of protein-protein interaction types we used basically two types only: Activation (or promotion) is denoted by →, while inhibition (or repression) is denoted by ⊣. Note that in the schematic views shown in Figure 3.4, Figure 3.6, and Figure 3.7 some nodes are repeated for readability.

In order to generate a boolean network model, we have preprocessed this model to gather the unique names since each node should be uniquely identified and all activators and inhibitors to each node should be gathered. Nodes represent proteins, compounds or

events in the pathway. After this process, the rules defined in the following section are applied to the model for translation to the input language of NuSMV.

A Boolean network is a directed graph composed of a set of Boolean variables as its nodes and a Boolean transfer function for each node. The state of each node in a BN can be either Active(1) or Not Active(0) at any time step, except for the nodes which correspond to the external (control) signal (CASP10, IAP, CASP3 elements in our case). The Boolean transfer function describes the transformation of the state of node *xi* from time t to $t + 1$, and it is built from the usual following logical connectives: $\vee$ (or, |), $\wedge$ (and, &), $\neg$ (not, !). In constructing the Boolean network for the Apoptosis pathways in Lung Cancers shown in Figure 3.4, Figure 3.6, and Figure 3.7, we used the methodology defined in the study of Gong et al. The state of a node is determined by its current state and that of its parents, which can be parental activators or parental inhibitors, that is,

$$x_i(t+1) = x_i(t) \vee Pa\, x_{act}\, Pa(t) \wedge \neg\, Pa\, x_{in}\, Pa(t) \qquad \text{EQUATION 1}$$

where $x_{act}\, Pa(t)$ and $x_{in}\, Pa(t)$ represent activators and inhibitors of the node x_i (Gong et al., 2011). For example, ATM can activate (phosphorylate) the p53 tumor suppressor, while the oncoprotein Bcl-2 can deactivate (dephosphorylate) p53. Then, the Boolean transfer function for *p53* is written as:

$$p53(t+1) = (p53(t) \vee ATM(t)) \wedge !Bcl\text{-}2(t) \qquad \text{EQUATION 2}$$

According to cancer studies, in normal cells, oncoproteins are strictly regulated by tumor suppressor proteins (Gong et al., 2011). Hence in Equation 1, the activators can change the state of a node only if no inhibitor is acting on that node, in the cancer cell model. It is known that the continuous activation of oncoproteins is very often caused by the loss of cell proliferation inhibitors (Gong et al., 2011).

4.2. SMV Translation of Apoptosis and Lung Cancer Model

Our goal is to investigate interesting behaviors of lung cancer cells. The apoptosis pathways in lung cancer are depicted in Figure 3.4, Figure 3.6 and Figure 3.7. Corresponding Boolean network comprises 58 variable nodes and the control nodes CASP10, IAP, CASP3, leading to 2^{58} possible states in the state-transition diagram. These 58 nodes are defined as Boolean variables as an example subset is shown in Figure 4.1.

In this section we present the rules applied for the NuSMV translation from the KEGG pathway model. Although we applied these rules to a specific model (Apoptosis pathways in Lung Cancer), we generalized the translation process with these rules. Hence, the translation process can be automatically applied to the other pathways defined in KEGG pathway database as future work.

```
VAR
    CASP3, CASP10, IAP: boolean;  // Control nodes
    // All other variables shown in Figure 3.4, 3.6, and 3.7
    Bax, Apaf, BclXL, Bcl2, p53, ... : boolean;
    Apoptosis: boolean;  // Events
    Degradation: boolean;
    DNA_Fragmentation: boolean;
    Stress_Signals: boolean;
    DNA_Damage: boolean;
```

Figure 4.1: Variable Definitions

Rule 1: *All variable nodes are defined as boolean in VAR section.*

In our BN model, the initial state of each node can be active (TRUE) or inactive (FALSE), except for the control nodes so that we can compare our results with several experiments. Not only proteins or molecules but also events such as Apoptosis, DNA Fragmentation or Stress Signals are defined as nodes as shown in Figure 4.2. Survival can be thought as the complement of Apoptosis but there are states such that the cell does enter neither Apoptosis nor Survival state. Hence Survival state is defined separately.

```
ASSIGN
    init(Apoptosis):={FALSE,TRUE};
    init(Degradation):={FALSE,TRUE};
    init(DNA_Fragmentation):={FALSE,TRUE};
    init(Stress_Signals):={FALSE,TRUE};
    init(DNA_Damage):={FALSE,TRUE};
    init(Survival):={FALSE,TRUE};
```

Figure 4.2: Variable Initializations

Rule 2: *All non control group variable nodes are initialized as {FALSE, TRUE} in ASSIGN section.*

The control group is defined as TRUE (active), since considerable over expression of these proteins or caspases has been documented in lung cancers with a very high frequency. If the control group variable is lost in vitro experiments it would be depicted as FALSE (deactivated) all the time.

```
init(CASP10) := {TRUE};
init(IAP) := {TRUE};
init(CASP3) := {TRUE};
```

Figure 4.3: Control Group Initializations

Rule 3: *All control group variable nodes are initialized as {FALSE} or {TRUE} if it is lost or over expressed with a high frequency in lung cancers respectively in ASSIGN section.*

The Figure 4.4 shows the independent variable nodes which means there is no activator or inhibitor arrow to these nodes. These variables are considered to be the inputs of our biological system. Their existence is independent from the rest of the system and the reactions in the system. Hence their values are assigned arbitrary as shown in Figure 4.4. This arbitrary assignment is an overapproximation of their behavior.

```
next(FasL) := FasL;
next(TRAIL) := TRAIL;
next(TNFAlfa) := TNFAlfa;
next(IL1) := IL1;
next(NGF) := NGF;
next(IL3) := IL3;
next(FLIP) := FLIP;
next(Bcl2XL) := Bcl2XL;
next(Apaf1) := Apaf1;
```

Figure 4.4: Update Rule of Independent Variables

Rule 4: *Update rule of* a*ll independent variable nodes which have no inhibitor or activator arrows are defined as itself.*

If a variable node has only activator arrows, only the activator node names are defined with logical operators and the default case is defined as the node's name itself. For example, the next value of Calpain is determined by the value of Ca2 existence only. If

Ca2 is activated then the Calpain node is activated too, if Ca2 is not activated, Calpain value shall remain same. The SMV code for Calpain is shown in Figure 4.5.

```
next(Calpain) :=
    case
                Ca2: TRUE;
                  TRUE: Calpain;
    esac;
```

Figure 4.5: State transition of Calpain

A similar example is DNA Fragmentation event. There are three activators of DNA Fragmentation which are DFF40, AIF or ENDOG activation. As mentioned above all these activators are combined with logical or operator. If at least one of these variables is activated then DNA Fragmentation event shall be executed.

```
next(DNA_Fragmentation) :=
    case
                (DFF40 | AIF | ENDOG): TRUE;
                                TRUE: DNA_Fragmentation;
    esac;
```

Figure 4.6: State transition of DNA Fragmentation

Rule 5: *Update rule of variable nodes which have only activator arrows is defined such that only activator node names are defined with logical or operators for true case condition and the default case is defined as node name itself.*

The compliment of Calpain state transition is applicable for NFKB since NFKB has only one inhibitor and has no activator as shown in Figure 3.6. Hence, only false condition is

defined for update rule of NFKB which depends on the IKBAlfa variable state. Default condition is again the state of the variable itself as shown in Figure 4.7.

```
next(NFKB) :=
    case
                IKBAlfa: FALSE;
                    TRUE: NFKB;
    esac;
```

Figure 4.7: State transition of NFKB

Rule 6: *Update rule of variable nodes which have only inhibitor arrows is defined such that only inhibitor node names are defined with logical or operators for false case condition and the default case is defined as node name itself.*

p53 plays a central role in Apoptosis. It is a tumor suppressor and it is activated when a DNA Damage is occurred. On the other hand Bcl2 existence causes inactivation of p53. Hence the next value of p53 variable is dependent both DNA Damage and Bcl2 variables. In this kind of situations activators of the variable node are combined with the inverse of the inhibitors by using logical and operator for the activation condition of the variable node. For inactivation condition inhibitors are combined with logical or operators. Default case is defined as the variable node itself.

```
next(p53) :=
    case
              DNA_Damage & !Bcl2: TRUE;
                          Bcl2: FALSE;
                            TRUE: p53;
    esac;
```

Figure 4.8: State transition of p53

Rule 7: *Update rule of variable nodes which have both activator and inhibitor arrows is defined such that only activator node names are defined with logical or operators and combined with the inverse of inhibitor node names for activation condition. For deactivation condition only inhibitor node names are combined with logical or operator. The default case is defined as node name itself.*

As stated in Rule 7, Apoptosis event is translated to SMV by combining the activators with logical or operators and combining compliment of inhibitors' states with logical operator and for true condition. False condition is determined by the combination of inhibitors' state.

```
next(Apoptosis) :=
    case
      (CASP3 | Bax | p53 | CASP6 | CASP7 | DNA_Fragmentation | Bad)
                & !(IAP | BclXL | Bcl2) : TRUE;
                    (IAP | BclXL | Bcl2): FALSE;
                                          TRUE: Apoptosis;
    esac;
```

Figure 4.9: State transition of Apoptosis

As stated in Rule 2, Survival state is not the exact complement of Apoptosis state. As seen in Figure 4.10 activators of Apoptosis state are not included in the update rule of Survival state, since their activation does not affect Survival directly.

```
next(Survival) :=
    case
                (IAP | BclXL | Bcl2 | Bad) : TRUE;
                                        TRUE: Survival;
    esac;
```

Figure 4.10: State transition of Survival

CHAPTER 5

PROPERTIES AND RESULTS

The biological queries a biologist can consider about the apoptosis are of different kinds. Several biological properties converted into CTL formulas have been discussed and temporal logic patterns are presented for cellular interaction networks in this chapter. We focus on the verification of properties similar to those in the study of Monteiro et al. (2008). Monteiro et al. developed patterns for biological questions (occurrence/reachability, pathway consequence, pathway sequence) by working with biologists. Gong et al. introduced examples of these patterns and they added sample queries in new categories, which are stable states, oscillation, and loop, on the study of Monteiro et al. We generalized the sample queries of Gong et al. as new patterns and combined with the pattern set presented in Monteiro et al. Hence all the categories we used in our study are generalized and became ready for the automation.

Table 5.1 shows the general structure of temporal queries we applied to our model in our study. Our specific CTL queries in the category of occurrence/reachability, pathway consequence, stable states, oscillation, loop, and pathway sequence in lung cancer are explained in this chapter.

Table 5-1: CTL Formula Patterns

Occurrence/Reachability pattern[10]	
It is possible for a state *P* to occur	*EF* (*P*)
It is necessary for a state *P* to occur	AF (*P*)
Consequence pattern[10]	
If a state *P* occurs, then it is possibly followed by a state *Q*	*AG* (*P*⇒*EF* (*Q*))
If a state *P* occurs, then it is necessarily followed by a state *Q*	*AG* (*P*⇒*AF* (*Q*))
Steady State pattern	
A state *P* can persist indefinitely	*AF(EG* (*P*))
A state *P* must persist indefinitely	*AF(AG* (*P*))
Oscillation pattern	
There is an oscillation in state *P*.	*AG* ((*P*⇒*AF* (!*P*)) & ((!*P*⇒*AF* (*P*)))
Loop pattern	
There is a positive feedback loop from state *P* to state *Q*.	*AG* ((*P*⇒*AF* (*Q*)) & ((*Q* ⇒*AF* (P)))
There is a negative feedback loop from state *P* to state *Q*.	*AG* ((*P*⇒*AF* (*Q*)) & ((*Q* ⇒*AF* (!*P*)))
Sequence pattern[10]	
A state *Q* is reachable and is possibly preceded all the time by a state *P*.	*E* (*P U Q*)
A state *Q* is necessarily preceded at some time by a state *P*. !E (!P U Q) pattern means the state Q is **necessarily** preceded at some time by a state P (Clarke et al., 1999). This CTL formula evaluates to true if and only if there is no path which satisfies Q,	!*E* (!*P U Q*)

[10] Monteiro et al., 2008

without satisfying P first where P and Q are atomic properties.	

5.1. Occurrence/Reachability

The very first question of a biologist about our cancer cell model is whether the cell can reach apoptosis or the survival of cancer cell is inevitable. In our study, these properties are classified as reachability and occurrence patterns respectively. We asked the following queries to our NuSMV model in order to learn possibility or indispensability of apoptosis and survival of the cancer cell.

Property 1: If CASP3, CASP10 and IAP (control nodes which are initialized as TRUE in our model) are overexpressed, the cancer cell will necessarily evade Apoptosis and will survive.

The corresponding CTL property is *AF(!Apoptosis & Survival)*.

This property holds in the model we have constructed for Apoptosis in Lung Cancer.

Property 2: If CASP3, CASP10 and IAP are overexpressed, is it possible for the cancer cell to reach the Apoptosis state?

The corresponding CTL property is *EF(Apoptosis)*.

This property is falsified in our model as expected. Falsification of Property 2 means it is not possible for the cancer cell to reach Apoptosis when the control variables are overexpressed. Since in lung cancer tumors overexpression of CASP3, CASP10 and IAP leads to increased cancer cell survival and decreased apoptosis rate (Shivapurkar et al., 2003), these two properties are consistent with the recent experimental results.

5.2. Consequence

Property 3: If Apaf-1 or CASP9 are overexpressed, i.e., (Apaf1 | CASP9) is true, the cell will necessarily reach a state satisfying (Apoptosis & !Survival) in the future.

The corresponding CTL property is *AG((Apaf1 | CASP9) & !IAP -> AF(Apoptosis & !Survival))*.

Property 3 is satisfied in our model. This result is consistent with the recent reports which claim Apaf-1 and CASP9 expression in lung cancer cell lines documented in the study of Soengas et al (1999). Continuous overexpression of Apaf-1 and CASP9 inhibit tumor formation in the absence of IAP. As explained in Property 1, in the presence of IAP, it is impossible to reach Apoptosis state according to our model.

Property 4: If p53 or CASP8 is continuously activated, the cell will eventually satisfy Apoptosis, that is, cell death is unavoidable.

The corresponding CTL property is *AG((p53 | CASP8) & !IAP → AF(Apoptosis))*.

Property 4 holds in our model. This result explains the two important tumor suppressors in lung cancer: p53 and CASP8. "These proteins may function as a tumor suppressor gene in neuroendocrine lung tumors" (Shivapurkar et al., 2003). It is known that p53 plays an important role in Apoptosis and its mutation is observed in many cancer types including lung (Soussi, 1996). CASP8 is known to play an obligatory role in apoptosis initiation by death receptors as explained in Section 3.1.1. Result of Property 4 supports these claims.

Property 5: If Bcl2 is continuously activated, the cell will necessarily evade Apoptosis.

The corresponding CTL property is *AG((Bcl2 & !IAP) -> AF(!Apoptosis & Survival))*.

Property 5 holds in our model. This result agrees with the experimental results about Bcl2 protein which explained in Section 3.2.1. Bcl2 is an anti-apoptotic protein and plays an important role in the prevention of Apoptosis (Brambilla et al., 1996; Chen et al., 1999). Again we suppressed the effect of IAP to be able see the direct effect of Bcl2 to Apoptosis and Survival process. As expected, our results showed that continuous activation of Bcl2 leads to survival of cancer cell.

Property 6: If Bcl2 is continuously activated, p53 which is the master regulator of the apoptosis is necessarily deactivated.

The corresponding CTL property is *AG(Bcl2 -> AF(!p53)).*

With this property we further investigated the role of Bcl2 in our model. NuSMV reported this property holds in the model. Bcl2 inhibits the activation of p53 as stated in the studies of Chen et al. (1999) and Kalomenidis et al. (2001). Sartorius and Krammer (2002) states that overexpression of Bcl2 is among the factors of chemotherapy resistance in human lung cancer cell lines. Our result is compatible with these experimental results which show that Bcl2 upregulation deregulates p53 protein leading to cancer cell survival each time.

Property 7: Another important gene IAP is queried for the role of it in Apoptosis and Survival.

The corresponding CTL properties are:

AG((!IAP) -> AF(Apoptosis))

AG((IAP) -> AF(Survival))

We found that these properties hold in the model. Property 7 proves that continuous deactivation of IAP leads to Apoptosis and continuous activation of this gene makes survival of cancer cell inevitable. As shown in several in vitro studies, IAP activation or deactivation has a major effect on Apoptosis and affects the response to chemotherapy in advanced lung cancer patients (LaCasse et al., 1998; Ferreira et al., 2001).

Property 8: Continuous MYC activation leads to the inhibition of Apoptosis.

The corresponding CTL property is *AG((MYC) -> AF(!Apoptosis)).*

Property 8 holds in our model. This result agrees with the discussion in the studies of Amati et al. (1998) and Shivapurkar et al. (2002). It is stated that MYC genes deregulation is frequently seen in lung cancer and in many forms of human cancer.

5.3. Steady States

Property 9: Are the states satisfied by the proposition (IAP & !Apoptosis) steady?

The corresponding CTL property is *AF (AG (IAP & !Apoptosis)).*

Property 9 holds in our model. This property shows that once the protein IAP is activated Apoptosis becomes relatively independent of other proteins' control and cell death cannot be performed. This property explains the reason why apoptosis is impossible when IAP control variable is activated as shown in Property 1.

Property 10: It is known that CASP3 loss is very frequent in lung and breast cancers (Shivapurkar et al., 2003). Therefore, is it the case that CASP3 is deactivated steadily?

The corresponding CTL property is *AF(AG(!CASP3)).*

In our model this property turns out to be satisfied. Sequential activation of downstream caspases such as CASP3, CASP8 etc. have an important role in the intrinsic pathway of apoptosis. Therefore continuous deactivation of the caspases are worthy of attention.

Property 11: Does deactivation of CASP8 ***can*** *persist indefinitely in our model.*

The corresponding CTL property is *AF (EG !CASP8)* .

As explained in Section 3.1.2 CASP8 may have an important role in Intrinsic pathway of Apoptosis. Since Apoptosis cannot be performed in cancer cell, we asked whether deactivation of CASP8 **can** persist indefinitely in our model.

Our results showed that this property is false which means CASP8 deactivation cannot persist indefinitely, in other words CASP8 certainly becomes activated at some time.

Property 12: Does the activation of NF-kB can persist indefinitely in our model.

The corresponding CTL property is *AF (EG NFKB)* .

According to Tang et. al. Nuclear Factor-kB (NF-kB) is frequently expressed in lung cancer (Tang et. al., 2006); therefore we checked this property on the model.

NuSMV falsified this property in our model. The reason is the absence of K-RAS pathways in our model which includes positive feedback loops for NF-kB activation. Actually, absence of some important pathways is the major difficulty in our study. Although we tried to include all related major pathways which affect the apoptosis process in lung cancer, there are still interactions which we did not include our scope. We had to limit our scope due to the state space explosion problem explained in Section 2.1.2. By limiting our scope, we are sacrificing some of the information which may affect our results as in this case. Persistent NF-kB activation is an expected result

however our model cannot satisfy this property due to the missing information about K-RAS pathways which lead to the activation of NF-kB.

5.4. Oscillation

Property 13: Does the release of control nodes may cause oscillations in the expression level of CASP8.

The corresponding CTL property is *AG((!CASP8 -> AF(CASP8)) & (CASP8 -> AF(!CASP8))).*

Our results showed that this CTL property is false which means there is no oscillation in the expression level of CASP8 in our model.

Similarly we examined the expression levels of p53, Bcl2, Bax and NFKB genes whether the negative feedback loops drive the oscillations in these genes localizations. In our cancer model, no oscillation is possible in the expression levels of p53, Bcl2, Bax and NFKB.

Property 14: Does IAP become activated again even if it is deactivated at some point.

The corresponding CTL property is *AG (!IAP -> AF IAP).*

The role of IAP activation is a major factor as shown in Section 5.1. Hence we wondered whether IAP becomes activated again though it is deactivated at some time. This property holds in our model.

Property 15: Does Bax become activated again after it is deactivated.

The corresponding CTL property is *AG (!Bax -> AF Bax).*

According to our results, this property is false. Unlike IAP, Bax is not activated once it is deactivated. It is reasonable since Bax is a pro-apoptotic protein as explained in Section 3.2.1. In a cancer cell, in contrast to Bcl2 increase, Bax expression decreases which enables cancer cell to evade apoptosis (Brambilla et al., 1996).

5.5. Loop

Property 16: NFKB can induce the transcription of IKBAlfa, while IKBAlfa is a negative regulator of NFKB.

The corresponding CTL property is *AG((IKBAlfa -> AF(!NFKB)) &(NFKB -> AF(IKBAlfa))).*

This property holds in our model as expected. With this property we verified a negative feedback loop.

5.6. Sequence

Property 17.1: Is activation of Bcl2 a necessary checkpoint for the cancer cell to evade apoptosis (i.e. the !Apoptosis state)?

The corresponding CTL property is *!E [!Bcl2 U !Apoptosis]*.

This property is false according to our results.

Property 17.2: We verified the same property for IAP and some other critical proteins which are known to regulate the Apoptosis process such as p53, Bax, AktPKB and, BclXL. However, none of them verified to be true. In particular, this means cancer cell survival is not dependent solely to the activation of neither Bcl2 nor any other protein such as IAP, p53, Bax, AktPKB and, BclXL.

Property 18: Is activation of IAP a possible checkpoint for the cancer cell to reach the (Survival) state?

The corresponding CTL property is *E [IAP U Survival]*.

This property is satisfied in our model which means cancer cell survival may preceded by the activation of IAP all the time. This shows the importance of this protein as explained in Section 3.2.2.

CHAPTER 6

CONCLUSION

In our study we modeled the apoptosis pathways in lung cancer by combining three signaling pathways from KEGG Database: Intrinsic and Extrinsic Apoptosis Pathways, P53 Signaling Pathway and p53 - DAP Kinase pathway in Lung cancers. We translated the constructed Boolean Network which is a combination of these three networks to the input language of NuSMV symbolic model checker. While performing translation, we determined the translation rules for automation of the translation process. Secondly we prepared specific queries for the control of the model we have constructed for Apoptosis process in lung cancers. We added generalized patterns of Steady States, Oscillation, Positive and Negative Feedback loops whose examples are given in the study of Gong et al. and combined them with the pattern set presented in the study of Monteiro et al. Hence all the patterns we used in our study are generalized for automation.

Contributions of our study are:

- Construction of the NuSMV model of Apoptosis process in lung cancers. As we explained before we combined three different signaling pathways in order to construct our model.

- Application of the model checking technique to query a biological system for comparing the experimental results with our model.
- Generalization of the rule set for translation to NuSMV input language from Boolean Network Model.
- Extension of the CTL property patterns used in biological studies and designing temporal properties from biological experiments.

Basic limitation of our study is the state space problem which is the common problem of model checking phenomenon. The reflection of this problem to our study is the limitation of signaling pathways which are included in our model. Although we tried to include all major pathways which affect the apoptosis process in lung cancer, there are many other pathways which affect the proteins included in our model. Some properties are falsified in our model due to the absence of these pathways but we cannot add all known pathways to our study. There is a huge amount of data about biological systems and most of the data is interconnected, in order to cope with the state space explosion problem we had to limit our scope. How to perform this abstraction in order to isolate a model by extracting it from the whole interacting biological system network in a systematic way is another research question. We performed the extraction process manually and automation of it can be handled in future studies.

Each CTL property increases the execution time and it is not possible to generate all CTL properties for corresponding experimental results which is another limitation of our study.

Future work for our study is the automation of the whole process described in this thesis. We have presented a systematic process so that any signaling pathway from KEGG Database (or representations in a similar form) can be automatically translated to NuSMV input language, selected type of queries can be automatically generated and checked for automatic model verification. Results of our study can be used for predictions in new models for lung cancer therapies after collaborating with biologists.

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APPENDICES

APPENDIX A. SMV MODEL OF APOPTOSIS PATHWAYS

```
MODULE main
  VAR
      FasL: boolean;
      Fas: boolean;
      TNFAlfa: boolean;
      TRAIL: boolean;
      IL1: boolean;
      NGF: boolean;
      IL3: boolean;
      TRAILR: boolean;
      TNFR1: boolean;
      IL1R: boolean;
      TrkA: boolean;
      IL3R: boolean;
      FADD: boolean;
      TRADD_FADD: boolean;
      TRADD_RIP1_TRAF2: boolean;
      MyD88_IRAK: boolean;
      PI3K: boolean;
      cAMP: boolean;
      NIK: boolean;
      IKK: boolean;
      AktPKB: boolean;
```

```
PKA: boolean;
Cn: boolean;
Calpain: boolean;
Ca2: boolean;
Bad: boolean;
NFKB: boolean;
IKBAlfa: boolean;
FLIP: boolean;
CytC: boolean;
Apaf1: boolean;
CASP9: boolean;
Bid: boolean;
CASP8: boolean;
CASP10: boolean;
IAP: boolean;
CASP6: boolean;
CASP3: boolean;
CASP7: boolean;
BclXL: boolean;
Bcl2: boolean;
Bax: boolean;
CASP12: boolean;
DFF45: boolean;
DFF40: boolean;
AIF: boolean;
ENDOG: boolean;
ATM: boolean;
p53: boolean;
DAPK: boolean;
p14ARF: boolean;
MDM2: boolean;
MYC: boolean;
PTEN: boolean;
Apoptosis: boolean;
Degradation: boolean;
DNA_Fragmentation: boolean;
```

```
    Stress_Signals: boolean;
    DNA_Damage: boolean;
    DNA_Methylation: boolean;
    Survival: boolean;


ASSIGN
  init(FasL) := {FALSE,TRUE};
  init(Fas) := {FALSE,TRUE};
  init(TNFAlfa) := {FALSE,TRUE};
  init(TRAIL) := {FALSE,TRUE};
  init(IL1) := {FALSE,TRUE};
  init(TRAILR) := {FALSE,TRUE};
  init(TNFR1) := {FALSE,TRUE};
  init(IL1R) := {FALSE,TRUE};
  init(TrkA) := {FALSE,TRUE};
  init(IL3R) := {FALSE,TRUE};
  init(FADD) := {FALSE,TRUE};
  init(TRADD_FADD) := {FALSE,TRUE};
  init(TRADD_RIP1_TRAF2) := {FALSE,TRUE};
  init(MyD88_IRAK) := {FALSE,TRUE};
  init(PI3K) := {FALSE,TRUE};
  init(cAMP) := {FALSE,TRUE};
  init(NIK) := {FALSE,TRUE};
  init(IKK) := {FALSE,TRUE};
  init(AktPKB) := {FALSE,TRUE};
  init(PKA) := {FALSE,TRUE};
  init(Cn) := {FALSE,TRUE};
  init(Calpain) := {FALSE,TRUE};
  init(Ca2) := {FALSE,TRUE};
  init(Bad) := {FALSE,TRUE};
  init(NFKB) := {FALSE,TRUE};
  init(IKBAlfa) := {FALSE,TRUE};
  init(FLIP) := {FALSE,TRUE};
  init(DAPK) := {FALSE, TRUE};
  init(CytC) := {FALSE,TRUE};
```

```
    init(Apaf1) := {FALSE,TRUE};
    init(CASP9) := {FALSE,TRUE};
    init(Bid) := {FALSE,TRUE};
    init(CASP8) := {FALSE,TRUE};
    init(CASP10) := {TRUE};
    init(IAP) := {TRUE};
    init(CASP6) := {FALSE,TRUE};
    init(CASP3) := {TRUE};
    init(CASP7) := {FALSE,TRUE};
    init(BclXL) := {FALSE,TRUE};
    init(Bcl2) := {FALSE,TRUE};
    init(Bax) := {FALSE,TRUE};
    init(CASP12) := {FALSE,TRUE};
    init(DFF45) := {FALSE,TRUE};
    init(DFF40) := {FALSE,TRUE};
    init(AIF) := {FALSE,TRUE};
    init(ENDOG) := {FALSE,TRUE};
    init(ATM) := {FALSE,TRUE};
    init(p53) := {FALSE,TRUE};
    init(MDM2) := {FALSE,TRUE};
    init(p14ARF) := {FALSE,TRUE};
    init(MYC) := {FALSE,TRUE};
    init(PTEN) := {FALSE,TRUE};

      init(Apoptosis):={FALSE,TRUE};
      init(Degradation):={FALSE,TRUE};
      init(DNA_Fragmentation):={FALSE,TRUE};
      init(Stress_Signals):={FALSE,TRUE};
      init(DNA_Damage):={FALSE,TRUE};
      init(Survival):={FALSE,TRUE};
      init(DNA_Methylation):={FALSE,TRUE};


next(FasL) := FasL;
next(TRAIL) := TRAIL;
next(TNFAlfa) := TNFAlfa;
```

```
next(IL1) := IL1;
next(NGF) := NGF;
next(IL3) := IL3;
next(FLIP) := FLIP;
next(MYC) := MYC;
next(PTEN) := PTEN;
next(DNA_Methylation) := DNA_Methylation;

next(Apaf1) :=
      case
                  DNA_Methylation: FALSE;
                     TRUE: Apaf1;
      esac;

next(DAPK) :=
      case
                  DNA_Methylation: FALSE;
                     TRUE: DAPK;
      esac;

next(p14ARF) :=
      case
            (DAPK | MYC) & !DNA_Methylation: TRUE;
                  DNA_Methylation: FALSE;
                     TRUE: p14ARF;
      esac;

next(MDM2) :=
      case
            p53 & !p14ARF & !AktPKB: TRUE;
                           p14ARF | AktPKB: FALSE;
                           TRUE: MDM2;
      esac;

next(Fas) :=
      case
```

```
            FasL: TRUE;
            TRUE: Fas;
      esac;

next(TRAILR) :=
      case
            TRAIL: TRUE;
             TRUE: TRAILR;
      esac;

next(TNFR1) :=
      case
            TNFAlfa: TRUE;
               TRUE: TNFR1;
      esac;

next(IL1R) :=
      case
            IL1: TRUE;
           TRUE: IL1R;
      esac;

next(TrkA) :=
      case
            NGF: TRUE;
            TRUE: TrkA;
      esac;

next(IL3R) :=
      case
            IL3: TRUE;
           TRUE: IL3R;
      esac;

next(FADD) :=
      case
```

```
            (Fas | TRAILR) & !FLIP: TRUE;
                                FLIP: FALSE;
                                TRUE: FADD;
      esac;

next(TRADD_FADD) :=
      case
            (IL1R | TNFR1) & !FLIP: TRUE;
                                FLIP: FALSE;
                                TRUE: TRADD_FADD;
      esac;

next(TRADD_RIP1_TRAF2) :=
      case
            TNFR1: TRUE;
             TRUE: TRADD_RIP1_TRAF2;
      esac;

next(MyD88_IRAK) :=
      case
            IL1R: TRUE;
            TRUE: MyD88_IRAK;
      esac;

next(PI3K) :=
      case
            (TrkA | IL3R): TRUE;
                     TRUE: PI3K;
      esac;

next(cAMP) :=
      case
            IL3R: TRUE;
            TRUE: cAMP;
      esac;
```

```
next(NIK) :=
      case
            (TRADD_FADD | MyD88_IRAK): TRUE;
                                 TRUE: NIK;
      esac;

next(IKK) :=
      case
            (NIK | AktPKB): TRUE;
                      TRUE: IKK;
      esac;

next(AktPKB) :=
      case
            PI3K & !PTEN: TRUE;
                  PTEN: FALSE;
                  TRUE: AktPKB;
      esac;

next(PKA) :=
      case
            cAMP: TRUE;
            TRUE: PKA;
      esac;

next(Bad) :=
      case
            Cn & !PKA & !AktPKB: TRUE;
                  (PKA | AktPKB): FALSE;
                        TRUE: Bad;
      esac;

next(IKBAlfa) :=
      case
            (IKK | NFKB): TRUE;
                    TRUE: IKBAlfa;
```

```
      esac;

next(NFKB) :=
      case
                  IKBAlfa: FALSE;
                    TRUE: NFKB;
      esac;

next(CASP10) :=
      case
            (TRADD_FADD | FADD): TRUE;
                        TRUE: CASP10;
      esac;

next(CASP8) :=
      case
                  (TRADD_FADD | FADD): TRUE;
                                  TRUE: CASP8;
      esac;

next(Bid) :=
      case
                  CASP8: TRUE;
                  TRUE: Bid;
      esac;

next(CytC) :=
      case
            (Bax | Bid | AIF) & !Bcl2 & !BclXL: TRUE;
                                  Bcl2 | BclXL: FALSE;
                                         TRUE: CytC;
      esac;

next(CASP9) :=
      case
                  (CytC & Apaf1) & !IAP & !AktPKB: TRUE;
```

```
                                    (IAP | AktPKB): FALSE;
                                                TRUE: CASP9;
      esac;

next(CASP6) :=
      case
                  CASP3 & !IAP: TRUE;
                        IAP: FALSE;
                        TRUE: CASP6;
      esac;

next(IAP) :=
      case
                  NFKB: TRUE;
                  TRUE: IAP;
      esac;

next(Bcl2) :=
      case
                  NFKB: TRUE;
                  TRUE: Bcl2;
      esac;

next(CASP3) :=
      case
            (CASP8 | CASP9 | CASP10 | CASP12) & !IAP: TRUE;
                                                IAP: FALSE;
                                                TRUE: CASP3;
      esac;

next(CASP7) :=
      case
                  (CASP8 | CASP9) & !IAP: TRUE;
                                    IAP: FALSE;
                                    TRUE: CASP7;
      esac;
```

```
next(DFF45) :=
      case
                  (CASP3 | CASP7) & !DFF40: TRUE;
                                    DFF40: FALSE;
                                    TRUE: DFF45;
      esac;

next(DFF40) :=
      case
                  DFF45: FALSE;
                  TRUE: DFF40;
      esac;

next(BclXL) :=
      case
                  NFKB & !Bad: TRUE;
                  Bad: FALSE;
                  TRUE: BclXL;
      esac;

next(AIF) :=
      case
                  Stress_Signals: TRUE;
                            TRUE: AIF;
      esac;

next(ENDOG) :=
      case
                  Stress_Signals: TRUE;
                            TRUE: ENDOG;
      esac;

next(Cn) :=
      case
                  Ca2: TRUE;
```

```
                  TRUE: Cn;
      esac;

next(Calpain) :=
      case
                  Ca2: TRUE;
                  TRUE: Calpain;
      esac;

next(CASP12) :=
      case
                  Calpain: TRUE;
                  TRUE: CASP12;
      esac;

next(Bax) :=
      case
                  (p53 | IL3R) & !IL3: TRUE;
                  IL3: FALSE;
                  TRUE: Bax;
      esac;

next(p53) :=
      case
                  DNA_Damage & !Bcl2 & !MDM2: TRUE;
                        Bcl2 | MDM2: FALSE;
                        TRUE: p53;
      esac;

next(Apoptosis) :=
      case
      (CASP3 | Bax | p53 | CASP6 | CASP7 | DNA_Fragmentation | Bad)
                  & !(IAP | BclXL | Bcl2)  : TRUE;
                  (IAP | BclXL | Bcl2): FALSE;
                                    TRUE: Apoptosis;
      esac;
```

```
next(Degradation) :=
      case
                  (IKBAlfa | DFF45): TRUE;
                        TRUE: Degradation;
      esac;

next(DNA_Fragmentation) :=
      case
                  (DFF40 | AIF | ENDOG): TRUE;
                  TRUE: DNA_Fragmentation;
      esac;

next(Survival) :=
      case
                  (IAP | BclXL | Bcl2 | Bad) : TRUE;
                  TRUE: Survival;
      esac;

SPEC EF(Apoptosis)

SPEC AF(!Apoptosis & Survival)

SPEC AG(Bcl2 -> AF(!Apoptosis & Survival))

SPEC AG(Bcl2 -> AF(!p53))

SPEC AF(AG(!CASP3))

SPEC AF(EG (!CASP8))

SPEC AF(EG (NFKB))

SPEC AG(((p53 | CASP8) & !IAP) -> AF(Apoptosis))

SPEC AG((!IAP) -> AF(Apoptosis))
```

```
SPEC AG((IAP) -> AF(Survival))

SPEC AG((MYC) -> AF(!Apoptosis))

SPEC AF(AG(IAP & !Apoptosis))

SPEC AG(((CASP8 -> EF(!CASP8)) & (!CASP8 -> EF(CASP8))))

SPEC AG((IKBAlfa -> AF(!NFKB)) &(NFKB -> AF(IKBAlfa)))

SPEC AG((Apaf1 | CASP9) & !IAP -> AF(Apoptosis & !Survival))

SPEC !E[!IAP U Survival]

SPEC E[NFKB U !Apoptosis]

SPEC E[IAP U Survival]

SPEC !E[!Bcl2 U !Apoptosis]

SPEC AG((!IAP) -> AF(IAP))

SPEC AG((!Bax) -> AF(Bax))
```

APPENDIX B. RESULTS OF NuSMV MODEL CHECKER

NuSMV 2.5.2 [11] is used for these results. This version of NuSMV is linked to the MiniSat SAT solver[12].

Result of Property 1:

-- specification AF (!Apoptosis & Survival) is true

Result of Property 2:

-- specification EF Apoptosis is false

-- as demonstrated by the following execution sequence

Trace Description: CTL Counterexample

Trace Type: Counterexample

-> State: 1.1 <-

FasL = FALSE

Fas = FALSE

TNFAlfa = FALSE

TRAIL = FALSE

IL1 = FALSE

NGF = FALSE

IL3 = FALSE

TRAILR = FALSE

TNFR1 = FALSE

IL1R = FALSE

[11] http://nusmv.fbk.eu

[12] http://www.cs.chalmers.se/Cs/Research/FormalMethods/MiniSat

TrkA = FALSE

IL3R = FALSE

FADD = FALSE

TRADD_FADD = FALSE

TRADD_RIP1_TRAF2 = FALSE

MyD88_IRAK = FALSE

PI3K = FALSE

cAMP = FALSE

NIK = FALSE

IKK = FALSE

AktPKB = FALSE

PKA = FALSE

Cn = FALSE

Calpain = FALSE

Ca2 = FALSE

Bad = FALSE

NFKB = FALSE

IKBAlfa = FALSE

FLIP = FALSE

CytC = FALSE

Apaf1 = FALSE

CASP9 = FALSE

Bid = FALSE

CASP8 = FALSE

CASP10 = TRUE

IAP = TRUE

CASP6 = FALSE

CASP3 = TRUE

CASP7 = FALSE

BclXL = FALSE

Bcl2 = FALSE

Bax = FALSE

CASP12 = FALSE

DFF45 = FALSE

DFF40 = FALSE

AIF = FALSE

ENDOG = FALSE

ATM = FALSE

p53 = FALSE

DAPK = FALSE

p14ARF = FALSE

MDM2 = FALSE

MYC = FALSE

PTEN = FALSE

Apoptosis = FALSE

Degradation = FALSE

DNA_Fragmentation = FALSE

Stress_Signals = FALSE

DNA_Damage = FALSE

DNA_Methylation = FALSE

Survival = FALSE

Result of Property 3:

-- specification AG ((Apaf1 | CASP9) & !IAP -> AF (Apoptosis & !Survival)) is true

Result of Property 4:

-- specification AG (((p53 | CASP8) & !IAP) -> AF Apoptosis) is true

Result of Property 5:

-- specification AG ((Bcl2 & !IAP) -> AF (!Apoptosis & Survival)) is true

Result of Property 6:

-- specification AG (Bcl2 -> AF !p53) is true

Result of Property 7:

-- specification AG (!IAP -> AF Apoptosis) is true

-- specification AG (IAP -> AF Survival) is true

Result of Property 8:

-- specification AG (MYC -> AF !Apoptosis) is true

Result of Property 9:

-- specification AF (AG (IAP & !Apoptosis)) is true

Result of Property 10:

-- specification AF (AG !CASP3) is true

Result of Property 11:

-- specification AF (EG !CASP8) is false

-- as demonstrated by the following execution sequence

Trace Description: CTL Counterexample

Trace Type: Counterexample

-> State: 2.1 <-

  FasL = TRUE

  Fas = FALSE

  TNFAlfa = FALSE

  TRAIL = FALSE

  IL1 = FALSE

  NGF = FALSE

  IL3 = FALSE

TRAILR = FALSE

TNFR1 = FALSE

IL1R = FALSE

TrkA = FALSE

IL3R = FALSE

FADD = TRUE

TRADD_FADD = FALSE

TRADD_RIP1_TRAF2 = FALSE

MyD88_IRAK = FALSE

PI3K = FALSE

cAMP = FALSE

NIK = FALSE

IKK = FALSE

AktPKB = FALSE

PKA = FALSE

Cn = FALSE

Calpain = FALSE

Ca2 = FALSE

Bad = FALSE

NFKB = FALSE

IKBAlfa = FALSE

FLIP = FALSE

CytC = FALSE

Apaf1 = FALSE

CASP9 = FALSE

Bid = FALSE

CASP8 = FALSE

CASP10 = TRUE

IAP = TRUE

CASP6 = FALSE

CASP3 = TRUE
CASP7 = FALSE
BclXL = FALSE
Bcl2 = FALSE
Bax = FALSE
CASP12 = FALSE
DFF45 = FALSE
DFF40 = FALSE
AIF = FALSE
ENDOG = FALSE
ATM = FALSE
p53 = FALSE
DAPK = FALSE
p14ARF = FALSE
MDM2 = FALSE
MYC = FALSE
PTEN = FALSE
Apoptosis = FALSE
Degradation = FALSE
DNA_Fragmentation = FALSE
Stress_Signals = FALSE
DNA_Damage = FALSE
DNA_Methylation = FALSE
Survival = FALSE
-> State: 2.2 <-
Fas = TRUE
CASP8 = TRUE
CASP3 = FALSE
DFF45 = TRUE
Survival = TRUE

-> State: 2.3 <-

Bid = TRUE

Degradation = TRUE

-- Loop starts here

-> State: 2.4 <-

CytC = TRUE

-> State: 2.5 <-

Result of Property 12:

-- specification AF (EG NFKB) is false

-- as demonstrated by the following execution sequence

Trace Description: CTL Counterexample

Trace Type: Counterexample

-> State: 3.1 <-

FasL = FALSE

Fas = FALSE

TNFAlfa = FALSE

TRAIL = FALSE

IL1 = FALSE

NGF = FALSE

IL3 = FALSE

TRAILR = FALSE

TNFR1 = FALSE

IL1R = FALSE

TrkA = FALSE

IL3R = FALSE

FADD = FALSE

TRADD_FADD = FALSE

TRADD_RIP1_TRAF2 = FALSE

MyD88_IRAK = FALSE

PI3K = FALSE

cAMP = FALSE

NIK = FALSE

IKK = FALSE

AktPKB = FALSE

PKA = FALSE

Cn = FALSE

Calpain = FALSE

Ca2 = FALSE

Bad = FALSE

NFKB = FALSE

IKBAlfa = FALSE

FLIP = FALSE

CytC = FALSE

Apaf1 = FALSE

CASP9 = FALSE

Bid = FALSE

CASP8 = FALSE

CASP10 = TRUE

IAP = TRUE

CASP6 = FALSE

CASP3 = TRUE

CASP7 = FALSE

BclXL = FALSE

Bcl2 = FALSE

Bax = FALSE

CASP12 = FALSE

DFF45 = FALSE

DFF40 = FALSE

AIF = FALSE

ENDOG = FALSE
ATM = FALSE
p53 = FALSE
DAPK = FALSE
p14ARF = FALSE
MDM2 = FALSE
MYC = FALSE
PTEN = FALSE
Apoptosis = FALSE
Degradation = FALSE
DNA_Fragmentation = FALSE
Stress_Signals = FALSE
DNA_Damage = FALSE
DNA_Methylation = FALSE
Survival = FALSE
-> State: 3.2 <-
CASP3 = FALSE
DFF45 = TRUE
Survival = TRUE
-- Loop starts here
-> State: 3.3 <-
Degradation = TRUE
-> State: 3.4 <-

Result of Property 13:

-- specification AG ((CASP8 -> EF !CASP8) & (!CASP8 -> EF CASP8)) is false
-- as demonstrated by the following execution sequence
Trace Description: CTL Counterexample
Trace Type: Counterexample
-> State: 4.1 <-

FasL = FALSE

Fas = FALSE

TNFAlfa = FALSE

TRAIL = FALSE

IL1 = FALSE

NGF = FALSE

IL3 = FALSE

TRAILR = FALSE

TNFR1 = FALSE

IL1R = FALSE

TrkA = FALSE

IL3R = FALSE

FADD = FALSE

TRADD_FADD = FALSE

TRADD_RIP1_TRAF2 = FALSE

MyD88_IRAK = FALSE

PI3K = FALSE

cAMP = FALSE

NIK = FALSE

IKK = FALSE

AktPKB = FALSE

PKA = FALSE

Cn = FALSE

Calpain = FALSE

Ca2 = FALSE

Bad = FALSE

NFKB = FALSE

IKBAlfa = FALSE

FLIP = FALSE

CytC = FALSE

Apaf1 = FALSE

CASP9 = FALSE

Bid = FALSE

CASP8 = FALSE

CASP10 = TRUE

IAP = TRUE

CASP6 = FALSE

CASP3 = TRUE

CASP7 = FALSE

BclXL = FALSE

Bcl2 = FALSE

Bax = FALSE

CASP12 = FALSE

DFF45 = FALSE

DFF40 = FALSE

AIF = FALSE

ENDOG = FALSE

ATM = FALSE

p53 = FALSE

DAPK = FALSE

p14ARF = FALSE

MDM2 = FALSE

MYC = FALSE

PTEN = FALSE

Apoptosis = FALSE

Degradation = FALSE

DNA_Fragmentation = FALSE

Stress_Signals = FALSE

DNA_Damage = FALSE

DNA_Methylation = FALSE

Survival = FALSE

Result of Property 14:

-- specification AG (!IAP -> AF IAP) is true

Result of Property 15:

-- specification AG (!Bax -> AF Bax) is false

-- as demonstrated by the following execution sequence

Trace Description: CTL Counterexample

Trace Type: Counterexample

-> State: 8.1 <-

FasL = FALSE

Fas = FALSE

TNFAlfa = FALSE

TRAIL = FALSE

IL1 = FALSE

NGF = FALSE

IL3 = TRUE

TRAILR = FALSE

TNFR1 = FALSE

IL1R = FALSE

TrkA = FALSE

IL3R = FALSE

FADD = FALSE

TRADD_FADD = FALSE

TRADD_RIP1_TRAF2 = FALSE

MyD88_IRAK = FALSE

PI3K = FALSE

cAMP = FALSE

NIK = FALSE

IKK = FALSE

AktPKB = FALSE

PKA = FALSE

Cn = FALSE

Calpain = FALSE

Ca2 = FALSE

Bad = FALSE

NFKB = FALSE

IKBAlfa = FALSE

FLIP = FALSE

CytC = FALSE

Apaf1 = FALSE

CASP9 = FALSE

Bid = FALSE

CASP8 = FALSE

CASP10 = TRUE

IAP = TRUE

CASP6 = FALSE

CASP3 = TRUE

CASP7 = FALSE

BclXL = FALSE

Bcl2 = FALSE

Bax = FALSE

CASP12 = FALSE

DFF45 = FALSE

DFF40 = FALSE

AIF = FALSE

ENDOG = FALSE

ATM = FALSE

p53 = FALSE

DAPK = FALSE
p14ARF = FALSE
MDM2 = FALSE
MYC = FALSE
PTEN = FALSE
Apoptosis = FALSE
Degradation = FALSE
DNA_Fragmentation = FALSE
Stress_Signals = FALSE
DNA_Damage = FALSE
DNA_Methylation = FALSE
Survival = FALSE
-> State: 8.2 <-
IL3R = TRUE
CASP3 = FALSE
DFF45 = TRUE
Survival = TRUE
-> State: 8.3 <-
PI3K = TRUE
cAMP = TRUE
Degradation = TRUE
-> State: 8.4 <-
AktPKB = TRUE
PKA = TRUE
-> State: 8.5 <-
IKK = TRUE
-- Loop starts here
-> State: 8.6 <-
IKBAlfa = TRUE
-> State: 8.7 <-

Result of Property 16:

-- specification AG ((IKBAlfa -> AF !NFKB) & (NFKB -> AF IKBAlfa)) is true

Result of Property 17.1:

-- specification !E [!Bcl2 U !Apoptosis] is false

-- as demonstrated by the following execution sequence

Trace Description: CTL Counterexample

Trace Type: Counterexample

-> State: 7.1 <-

FasL = FALSE

Fas = FALSE

TNFAlfa = FALSE

TRAIL = FALSE

IL1 = FALSE

NGF = FALSE

IL3 = FALSE

TRAILR = FALSE

TNFR1 = FALSE

IL1R = FALSE

TrkA = FALSE

IL3R = FALSE

FADD = FALSE

TRADD_FADD = FALSE

TRADD_RIP1_TRAF2 = FALSE

MyD88_IRAK = FALSE

PI3K = FALSE

cAMP = FALSE

NIK = FALSE

IKK = FALSE

AktPKB = FALSE

PKA = FALSE

Cn = FALSE

Calpain = FALSE

Ca2 = FALSE

Bad = FALSE

NFKB = FALSE

IKBAlfa = FALSE

FLIP = FALSE

CytC = FALSE

Apaf1 = FALSE

CASP9 = FALSE

Bid = FALSE

CASP8 = FALSE

CASP10 = TRUE

IAP = TRUE

CASP6 = FALSE

CASP3 = TRUE

CASP7 = FALSE

BclXL = FALSE

Bcl2 = FALSE

Bax = FALSE

CASP12 = FALSE

DFF45 = FALSE

DFF40 = FALSE

AIF = FALSE

ENDOG = FALSE

ATM = FALSE

p53 = FALSE

DAPK = FALSE

p14ARF = FALSE

MDM2 = FALSE

MYC = FALSE

PTEN = FALSE

Apoptosis = FALSE

Degradation = FALSE

DNA_Fragmentation = FALSE

Stress_Signals = FALSE

DNA_Damage = FALSE

DNA_Methylation = FALSE

Survival = FALSE

Result of Property 17.2:

-- specification !E [!IAP U Survival] is false

-- as demonstrated by the following execution sequence

Trace Description: CTL Counterexample

Trace Type: Counterexample

-> State: 5.1 <-

FasL = FALSE

Fas = FALSE

TNFAlfa = FALSE

TRAIL = FALSE

IL1 = FALSE

NGF = FALSE

IL3 = FALSE

TRAILR = FALSE

TNFR1 = FALSE

IL1R = FALSE

TrkA = FALSE

IL3R = FALSE

FADD = FALSE

TRADD_FADD = FALSE

TRADD_RIP1_TRAF2 = FALSE

MyD88_IRAK = FALSE

PI3K = FALSE

cAMP = FALSE

NIK = FALSE

IKK = FALSE

AktPKB = FALSE

PKA = FALSE

Cn = FALSE

Calpain = FALSE

Ca2 = FALSE

Bad = FALSE

NFKB = FALSE

IKBAlfa = FALSE

FLIP = FALSE

CytC = FALSE

Apaf1 = FALSE

CASP9 = FALSE

Bid = FALSE

CASP8 = FALSE

CASP10 = TRUE

IAP = TRUE

CASP6 = FALSE

CASP3 = TRUE

CASP7 = FALSE

BclXL = FALSE

Bcl2 = FALSE

Bax = FALSE

CASP12 = FALSE

DFF45 = FALSE

DFF40 = FALSE

AIF = FALSE

ENDOG = FALSE

ATM = FALSE

p53 = FALSE

DAPK = FALSE

p14ARF = FALSE

MDM2 = FALSE

MYC = FALSE

PTEN = FALSE

Apoptosis = FALSE

Degradation = FALSE

DNA_Fragmentation = FALSE

Stress_Signals = FALSE

DNA_Damage = FALSE

DNA_Methylation = FALSE

Survival = TRUE

Result of Property 18:

-- specification E [IAP U Survival] is true