Symbolic Model Checking of Signaling Pathways in Pancreatic Cancer

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Abstract

Model Checking is a formal verification method widely used for the automated verification and analysis of hardware systems and digital circuits. In this work, we apply Model Checking to the study of a biological system – the HMGB1 Boolean network. Recent studies on pancreatic cancer cells have found that the overexpression of HMGB1, a DNA-binding protein, can decrease apoptosis (programmed cell death) and increase cancer cell survival. Also, knocking out HMGB1 or its receptors can increase apoptosis in cancer cells. In this paper, we first build a single-cell, Boolean network to model the crosstalk of three signaling pathways activated by HMGB1. Then, we apply Model Checking to formally query and verify some desired temporal logic properties of the HMGB1 model. 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.

Keywords: Model Checking, HMGB1, Signaling Pathway, Boolean Network, Pancreatic Cancer

1 Introduction

The High-mobility group box-1 (HMGB1) protein is a DNA-binding nuclear protein, present in almost all eukaryotic cells [1, 2]. It is released in response to cell injury or during cell death. The expression level of HMGB1 has been found to be elevated in many tumors [3, 4, 5]. Recent *in vitro* studies with pancreatic cancer cells [6] showed that the targeted knockout or inhibition of HMGB1 or its receptors could increase apoptosis and suppress cancer cell growth.

In [7, 8], we proposed the first rule-based, computational model of the HMGB1 signaling pathway, and simulated it via ordinary differential equations and Gillespie's stochastic simulation algorithm. Furthermore, we applied statistical model checking to investigate the tumorigenesis induced by HMGB1. Our simulations have successfully explained recent experimental results involving HMGB1 and pancreatic cancer, and confirmed the importance of stochasticity and discretization effects [9, 10]. In particular, our model predicted a dose-dependent P53 (a tumor suppressor protein) and Cyclin E (a cell cycle regulatory protein) response curve to increasing HMGB1 stimulus. This behavior could be tested by the future experiments. However, since the rule-based model contains many undetermined free parameters, the verification power of statistical model checking is hampered when more molecular components and reactions are included.

An alternative approach to depict the signaling pathway is Boolean network (BN) modeling. A BN is a coarse-grained abstraction of a dynamic system, and has been previously applied to gene regulatory network and signaling pathway studies [11, 12]. In a BN, each node is allowed only two possible states, either "active" (ON) or "not active" (OFF), and each internal node is updated on the basis of the state of the nodes feeding into it. Given a BN model, one of the system 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 [13]. Model Checking is a technique that can be used to solve this problem.

Model Checking [14] is an automated verification technique which has been developed for verifying models of hardware, digital circuits, and software designs. Given a model M of a system (usually expressed as a state-transition diagram), a set of starting states S_0, and a specification (or property) described by a temporal logic formula ϕ, the Model Checking problem is to determine whether the model M meets the desired specification ϕ, that is to determine whether $M, S_0 \models \phi$. Model Checking has been successfully applied to formally verify a variety of finite-state, deterministic and stochastic systems. It has two unique capabilities compared with other techniques: first, the algorithms can exhaustively search the state space of the concurrent system to determine the truth of specification; second, the algorithms can give a counterexample to the desired specification if the property does not hold. A number of formal verification studies [15], including our recent work related to statistical model checking [7, 8], demonstrated that model checking is a powerful technique for ver-

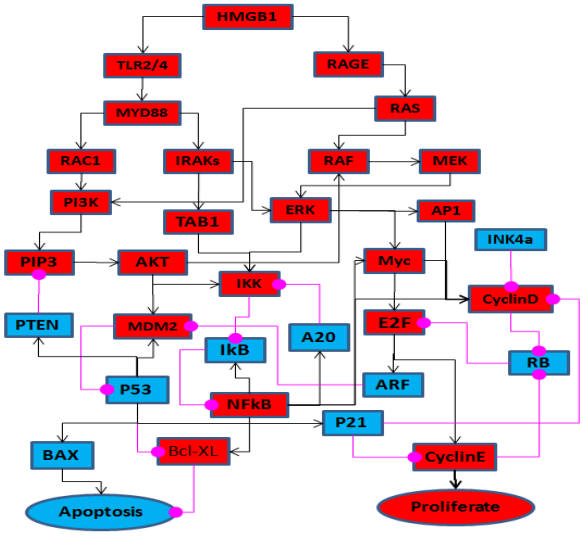

Figure 1: Schematic view of HMGB1 signal transduction, blue nodes represent tumor-suppressor proteins, red one represents oncoproteins/lipids. Arrow → represents protein activation, circle-headed arrow ⊸ represents deactivation.

ifying temporal logic properties of biological networks.

In this work, we first construct a Boolean network model of the HMGB1 signal transduction to describe the crosstalk among different signaling pathways. Then, we briefly describe the temporal logic Computation Tree Logic (CTL). Finally, we code a number of important behavioral properties as CTL formulae and check them on the BN model, and compare the results with some *in vitro* experiments. To the best of the authors' knowledge, this work is the first attempt to apply standard Model Checking to study the HMGB1 Boolean network signaling pathway.

2 The HMGB1 Boolean Network Model

A number of experiments have found that HMGB1 could activate at least three signaling pathways: the PI3K-P53, NFκB and RAS-RB pathways. These pathways play an important role in tumor growth and inflammation, through binding and activation of different receptors, such as receptors for advanced glycation end products (RAGEs) and toll-like receptors (TLRs2/4).

In Figure 1 we present the Boolean network model of the HMGB1 signaling pathway that has been discussed in our recent works [7, 8]. We denote activation (or promotion) by →, while inhibition (or repression) is denoted by ⊸ or ⊣. Here, we will briefly reiterate the PI3K-P53, NFκB and RAS-RB signaling pathways and their association with apoptosis and cell proliferation.

PI3K-P53 pathway: $PI3K \rightarrow PIP3 \rightarrow AKT \rightarrow MDM2 \dashv P53 \rightarrow BAX \rightarrow Apoptosis$. The HMGB1 protein secreted from the nucleus can activate one of its receptors TLR2/4, leading to the activation of the proteins MYD88, RAC1 and PI3K. In turn, PI3K phosphorylates the lipid PIP2 to PIP3, which can subsequently activate AKT. After being phosphorylated by AKT, the activated MDM2, one of P53's transcription targets, will translocate into the nucleus to inhibit P53's transcriptional activity [16]. The protein P53 is one of the most important tumor suppressor proteins, and its mutation occurs in over 50% of pancreatic cancers [17]. The activation of P53 will induce the transcription of P21, which can inhibit the activity of many regulatory proteins including Cyclin D/E - leading to cell cycle arrest - and BAX, which can initiate apoptosis. Moreover, P53 regulates the transcription of PTEN [18], one of the most commonly lost tumor suppressors in human pancreatic cancer. The protein PTEN can hydrolyze PIP3 to PIP2, thereby inhibiting the activation of AKT and MDM2, resulting in a positive feedback loop: $P53 \rightarrow PTEN \dashv PIP3 \rightarrow AKT \rightarrow MDM2 \dashv P53$.

NFκB pathway: $IKK \dashv I\kappa B \dashv NF\kappa B \rightarrow BclXL \dashv Apoptosis$. The activation of TLR2/4 by HMGB1 could also stimulate the NFκB pathway through signaling via the MyD88, IRAKs and TAB1 proteins to activate the IκB kinase (IKK). In resting normal cells, NFκB resides in the cytoplasm, where it is bound to and inhibited by IκB. Once activated, IKK will phosphorylate and deactivate IκB, leading to the release of NFκB – an important transcription factor. Then, the free NFκB will translocate into the nucleus to promote the transcription of a number of genes, including Cyclin D and the anti-apoptotic protein Bcl-XL. Experiments have confirmed that A20 and IκB are also NFκB's transcription targets [19, 20]. The newly synthesized IκB inhibits NFκB's transcriptional machinery by binding to NFκB in the nucleus and taking it out to the cytoplasm. Also, the newly synthesized A20 can inhibit NFκB by inhibiting IKK's activity, leading to two negative feedback loops: $NF\kappa B \rightarrow I\kappa B \dashv NF\kappa B$, and $NF\kappa B \rightarrow A20 \dashv IKK \dashv I\kappa B \dashv NF\kappa B$.

RAS-RB pathway: $RAS \rightarrow RAF \rightarrow MEK \rightarrow ERK \rightarrow CyclinD \dashv RB \dashv E2F \rightarrow CyclinE \rightarrow S\ phase$. HMGB1 can activate another receptor, RAGE, to initiate a cascade of reactions including the activation of the RAS, RAF, MEK and ERK proteins. The activated ERK will phosphorylate several transcription factors, for example AP1, which activate the expression of a number of regulatory proteins, including Cyclin D, enabling progression of the cell cycle through the G1 phase. E2F is another important transcription factor for many cell-cycle regulatory proteins [21] and the tumor suppressor protein ARF. In resting cells, the unphosphorylated RB - a tumor suppressor - binds to E2F to inhibit its transcriptional activity. E2F will be activated when its inhibitor RB is phosphorylated and inhibited by Cyclin D, inducing the transcription of Cyclin E and Cyclin-dependent protein kinases (CDK2) which promote cell cycle progression to the S phase. Cyclin E, in turn, continues to inhibit the activity of RB, leading to a forward

positive feedback loop [22, 23]. The oncoprotein K-ras (a member of the RAS protein family), and two tumor suppressor proteins, INK4a which inhibits CyclinD-CDK4/6's activity (only Cyclin D is shown in Fig.1), and ARF which can inhibit MDM2's activity to stabilize P53's expression level, are mutated in up to 90% of pancreatic cancers [17].

Boolean Modeling: A Boolean network is composed of a graph G and a Boolean transfer function for each node. The state of each node in a BN can be either ON(1) or OFF(0) at any time step, except for the nodes which correspond to the external (control) signal (HMGB1 in Fig. 1). The Boolean transfer function describes the transformation of the state of node x_i from time t to $t+1$, and it is built from the usual Boolean connectives: $\vee$ (or, |), $\wedge$ (and, &), $\neg$ (not, !). We adopt the qualitative dynamical systems methodology proposed in [24, 25]. The state of a node at the next time step depends on its current state and that of its parents, which can be parental activators or parental inhibitors, that is,

$$x_i(t+1) = \Big(x_i(t) \vee \bigvee_{Pa} x^a_{Pa}(t)\Big) \wedge \neg\Big(\bigvee_{Pa} x^{in}_{Pa}(t)\Big) \qquad (1)$$

where $x^a_{Pa}(t)$ and $x^{in}_{Pa}(t)$ represent activators and inhibitors of the node x_i. For example, PI3K can activate (phosphorylate) the lipid PIP2 to PIP3, while the tumor suppressor PTEN can deactivate (dephosphorylate) PIP3 to PIP2. Then, the boolean transfer function for $PIP3$ is written as $PIP3(t+1) = (PIP3(t) \vee PI3K(t)) \wedge !PTEN(t)$.

It is known that in normal cells, oncoproteins are strictly regulated by tumor suppressor proteins. With Eq. (1) we mean that in our cancer cell model, the activators can change the state of a node only if no inhibitor is acting on that node. This is in accord with evidence from cancer studies: the continuous activation of oncoproteins is very often caused by the loss of cell proliferation inhibitors. In a BN model, it means that such tumor suppressor proteins are in OFF state.

3 Model Checking

Let $AP = \{p, q, r, ...\}$ be a set of atomic propositions (predicates) defined over a finite set of states S. A Kripke structure $M = (S, s_0, R, L)$ represents a finite-state concurrent system, where $s_0 \in S$ is an initial state, R is a transition relation between states, and $L : S \to 2^{AP}$ is a labeling function that labels each state with the set of atomic propositions true in that state. Given a temporal logic formula ϕ expressing some desired property, the Model Checking problem [14] is to find the set of all states in S that satisfy ϕ: $\{s \in S | M, s \models \phi\}$.

3.1 Computation Tree Logic

A Computation Tree Logic (CTL) formula describes a property of computation trees [14]. The root of a computation tree corresponds to the set of initial states, while the other nodes on the tree correspond to all possible computation paths from the root. A CTL formula is constructed from a set of atomic propositional variables AP, Boolean logic connectives including $\to$ (implication), *temporal* operators describing properties of a path, and *path* quantifiers.

The temporal operators are: $\mathbf{X}\psi$ – ψ holds in the next state of the path; $\mathbf{F}\psi$ – ψ holds at some state in the future (eventually) on the path; $\mathbf{G}\psi$ – ψ holds globally (always) at every state on the path; $\psi_1\mathbf{U}\psi_2$ – ψ_1 holds until ψ_2 holds. There are two path quantifiers which describe the branching structure in the computation tree: $\mathbf{A}\phi$ – for *all* paths ϕ holds, and $\mathbf{E}\phi$ – there *exists* a path for which ϕ holds. An example of a CTL property is $\mathbf{AG}(Req \to \mathbf{AF}\ Ack)$, meaning that if a *Request* occurs, then it will be eventually *Acknowledged*.

There are two types of CTL formulas: state formulas ψ and path formulas ϕ. A state formula has a truth value at a specific state, while a path formula has a truth value along a specific (computation) path. The syntax of CTL is the following:

$$\psi ::= AP \mid \psi_1 \vee \psi_2 \mid \neg\psi \mid \mathbf{E}\phi \mid \mathbf{A}\phi$$
$$\phi ::= \mathbf{X}\psi \mid \mathbf{F}\psi \mid \mathbf{G}\psi \mid \psi_1\mathbf{U}\psi_2.$$

A path π in a Kripke structure $M = (S, s_0, R, L)$ is an infinite sequence of states, $\pi = s_0, s_1, \ldots$, where $s_i \in S$, and for every $i \geq 0$, $(s_i, s_{i+1}) \in R$. We use π^i to denote the suffix of π starting at the i-th state. The semantics of CTL is defined below (the interested reader can find more details in [14]):

$M, s \models p$	iff $p \in L(s)$;
$M, s \models \neg\psi$	iff $M, s \models \psi$ does not hold
$M, s \models \psi_1 \vee \psi_2$	iff $M, s \models \psi_1$ or $M, s \models \psi_2$;
$M, \pi \models \mathbf{X}\psi$	iff $M, \pi^1 \models \psi$;
$M, \pi \models \psi_1\mathbf{U}\psi_2$	iff there exists $k \geq 0$ such that $M, \pi^k \models \psi_2$ and for all $0 \leq j < k$, $M, \pi^j \models \psi_1$;
$M, s \models \mathbf{E}\phi$	iff there exists a path π from s such that $M, \pi \models \phi$;
$M, s \models \mathbf{A}\phi$	iff for every path π from s, $M, \pi \models \phi$.

The temporal operators $\mathbf{F}$ and $\mathbf{G}$ can be defined as $\mathbf{F}\psi = true\,\mathbf{U}\,\psi$ and $\mathbf{G}\psi = \neg\mathbf{F}\neg\psi$.

3.2 CTL Model Checking

The Symbolic Model Verifier (SMV) [26] was developed at Carnegie Mellon University in the early 90's. SMV is the first model checker for CTL based on binary decision diagram (BDD)[27], a data structure that can be used to efficiently represent Boolean functions. The SMV language provides a platform to describe general state transition diagrams, and Boolean networks in particular. To verify CTL formulas over SMV models one can use the original SMV tool or NuSMV [28]. The output of the model checker

```
MODULE MAIN
VAR
        HMGB1: boolean;
        PI3K, PIP3, PTEN, ... : boolean;
ASSIGN
        init(PI3K):={0,1};  init(PTEN):={0,1};
        init(PIP3):={0,1};  init(INK4a):=0; ...
        next(PIP3):=    //update rule for PIP3
          case
          PI3K & !PTEN: 1;
                PTEN: 0;
                   1: PIP3;
        ... ...       //update rules for other variables
SPEC  AG(RAS → AF(CyclinE)); // property verification
```

Figure 2: SMV code for the HMGB1 Boolean Network

could be either "true" (property satisfied) or a counterexample trace showing why the property is false.

In Figure 2 we report a portion of the SMV code for the HMGB1 Boolean network model as an example. The code can be divided into three parts: variable declarations ("boolean" in Figure 2); initialization of the states for each variable with "**init**"; implementation – updating the state of each node in the state transition diagram with "**next**". The verification of CTL properties is encoded using the "**SPEC**" statement.

4 Applications and Results

Our goal is to investigate interesting behaviors of pancreatic cancer cells. The HMGB1 Boolean network depicted in Fig. 1 comprises 33 variable nodes and the control node HMGB1, leading to 2^{33} possible states in the state-transition diagram. In order to compare our results with several *in vitro* experiments, in our BN model the initial state of each node can be active (1) or inactive (0), except for the control node (HMGB1) and the proteins RAS, P53 and INK4a, since those are either mutated or lost with a very high frequency in pancreatic cancer. Therefore, we set INK4a and P53 to be OFF (0), and RAS to be ON (1) initially. The SMV code is available online: http://www.cs.cmu.edu/~haijung/research/HMGB1.smv.

Several rules for the translation of biological patterns (properties) into CTL formulas have been discussed in [15, 29]. We focus on the verification of five types of properties similar to those in [29]: *occurrence/reachability, consequence, steady states, oscillation/loop*, and *sequence/pathway*.

I: Occurrence/Reachability

Property 1: If HMGB1 is overexpressed, the cancer cell will **necessarily** activate *Proliferate* at some time in the future. The following CTL property is verified:

$$\mathbf{AF}(\textit{Proliferate}) : \textit{True}$$

Property 2: If HMGB1 is overexpressed, is it **possible** that the cancer cell will reach the *Apoptosis* state? The following CTL property is proved to be false:

$$\mathbf{EF}(\textit{Apoptosis}) : \textit{False}$$

These two properties are consistent with the recent experimental discoveries that the elevated expression of HMGB1 leads to increased cancer cell survival and decreased apoptosis in pancreatic cancer cells [6].

II: Consequence

Property 3: If HMGB1's receptors TLR or RAGE are overexpressed, *i.e.*, $(TLR|RAGE)$ is true, the cell will **necessarily** reach a state satisfying (!*Apoptosis* & *Proliferate*) in the future. The following CTL property is true:

$$\mathbf{AG}\{(TLR|RAGE) \rightarrow \mathbf{AF}(!\textit{Apoptosis} \,\&\, \textit{Proliferate})\}$$

Property 3 is consistent with the recent experimental results in [6] – the overexpression of RAGE is associated with pancreatic cancer cell proliferation.

Property 4: If RAS is continuously activated, the cell will eventually satisfy (!*Apoptosis* & *Proliferate*), that is, cell proliferation is unavoidable. The following CTL property is true:

$$\mathbf{AG}\{(\textit{RAS}) \rightarrow \mathbf{AF}(!\textit{Apoptosis} \,\&\, \textit{Proliferate})\}$$

Property 4 explains an important phenomenon in cancer: KRAS mutation exists in over 90% of pancreatic adenocarcinomas [17], thereby leading to a continuous activation of the mitogen-activated protein kinase (MAPK) pathway which promotes cell proliferation.

III: Steady States

Property 5: Are the states satisfied by the proposition (!*P*53 & !*Apoptosis* & *CyclinE* & *Proliferate*) steady? The following CTL property is true:

$$\mathbf{AF}\{\mathbf{AG}(!P53 \,\&\, !\textit{Apoptosis} \,\&\, \textit{CyclinE} \,\&\, \textit{Proliferate})\}$$

This property shows that once the protein Cyclin E is activated and DNA synthesis has commenced, the cell will go to S-phase and stay in the *Proliferate* state. Cell growth thus becomes relatively independent of external controls and can not be stopped.

Property 6: It is known that RB is very frequently deactivated in most types of cancer. Therefore, is it the case that RB is (eventually) steadily OFF? In our cancer cell model this turns out to be true:

$$\mathbf{AF}\{\mathbf{AG}(!RB)\} : \textit{True}$$

IV: Oscillation/Loop
Property 7: The release of HMGB1 will cause oscillations in the expression level of NFκB. The following CTL property is verified to be true:

$$\mathbf{AG}\{(!NF\kappa B \rightarrow \mathbf{AF}(NF\kappa B)) \,\&\, (NF\kappa B \rightarrow \mathbf{AF}(!NF\kappa B))\}$$

Property 7 agrees with Hoffmann's population-level experiment in which damped oscillations in NFκB localization were observed after HMGB1 stimulation [20]. This is due to the fact that the NFκB signaling pathway is regulated by two negative feedback loops that drive the oscillations in NFκB nuclear-cytoplasmic localization.
Property 8: We also verified a negative feedback loop, for example, P53 can induce the transcription of MDM2, while MDM2 is a negative regulator of P53. The following property is true:

$$\mathbf{AG}\{(P53 \rightarrow \mathbf{AF}(MDM2)) \,\&\, (MDM2 \rightarrow \mathbf{AF}(!P53))\}$$

V: Sequence/Pathway
Property 9: Is NFκB's activation a **necessary** checkpoint that the cancer cell should go through before it reaches (!*Apoptosis* & *Proliferate*)? We verify the CTL property:

$$!\mathbf{E}\{(!NF\kappa B)\ \mathbf{U}\ (!Apoptosis \,\&\, Proliferate)\}$$

To better understand the meaning of this property consider the formula:

$$!\mathbf{E}\{!a\ \mathbf{U}\ b\}$$

where a, b are atomic propositions. This formula is true if and only if there is no path in which b is satisfied without satisfying a first. In other words, b is necessarily preceded by a. Property 9 is verified to be *false* in our model. In particular, this means that cancer proliferation is possible even without NFκB's activation.

5 Conclusions

We have presented and verified a Boolean network model for the HMGB1 signaling pathway in pancreatic cancer. Five types of properties associated with *Apoptosis* and *Proliferation* were formally expressed in the temporal logic CTL and verified by Symbolic Model Checking. Our results show that the Boolean network model can capture the most important characteristics of the HMGB1 signaling pathway. In particular, if HMGB1 is overexpressed, the cell will be necessarily reach the *Proliferation* state, but it cannot reach *Apoptosis*. Some properties could also provide new insights into the cancer therapies. For example, targeting the NFκB and ERK pathways may lead to programmed cell death and decrease the proliferation and metastasis ability of cancer cells. With Symbolic Model Checking one could also study behaviors which are difficult to simulate in models based on differential equations, or via stochastic simulation. For example, the sequence/pathway property in signal transduction will help to verify that some sequence will drive the network to a pre-specified state. Coupled with expressive CTL formulas, formal verification techniques have become a promising tool to develop and validate models of signaling pathways.

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