2011 50th IEEE Conference on Decision and Control and
European Control Conference (CDC-ECC)
Orlando, FL, USA, December 12-15, 2011

Formal Analysis for Logical Models of Pancreatic Cancer

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Abstract— **We apply formal verification techniques for studying the behavior of signaling pathways important in cancer. In particular, we use Model Checking for verifying behavioral properties of a single-cell, *in silico* model of pancreatic cancer. We are interested in properties associated with apoptosis (programmed cell death), cell cycle arrest and proliferation. The properties are specified in temporal logics and include, for example, whether there are checkpoints that the cancer cell should go through before it reaches a given state. Our model includes several major signaling pathways, including the Hedgehog, WNT, KRAS, RB-E2F, NFkB, p53, TGFβ, and apoptosis pathways, which have been recently found to be mutated frequently in pancreatic cancer. The model is formally analyzed via symbolic Model Checking, and shown to agree well qualitatively with experiments. We conclude that Model Checking offers a powerful approach for studying logical models of relevant biological processes.**

I. INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive malignancy and the 4^{th} leading cause of cancer-related death in the United States. It arises from intraepithelial neoplasia (PanIN), a progression of lesions that occur in the pancreatic ducts. It is characterized by a propensity for early local and distant invasion – rapid growth, early metastasis – and an unresponsiveness to most conventional treatments – it is highly resistant to chemotherapy and radiation. Vogelstein *et al.*'s [1] global genomic analysis identified 12 cellular signaling pathways that are genetically altered in over 67% of pancreatic cancers. The study also found that PDAC contains an average of 63 genetic alterations, and that the KRAS, apoptosis, TGF-β, Hedgehog, and Wnt/Notch signaling pathways, and the regulation of G1/S phase transition have genetic alterations in 100% of tumors. A number of molecular and pathological analyses of evolving pancreatic adenocarcinoma revealed progressive genetic mutations of KRAS, CDKN2A, TP53, SMAD4, corresponding to the mutations of the KRAS, INK4a, ARF, P53, and SMAD4 proteins in the above mentioned pathways. Mutations of oncoproteins and tumor suppressor proteins result in uncontrolled cell proliferation and evasion of apoptosis (programmed cell death), eventually leading to cancer. In addition, PDAC overexpresses a number of growth factors (GF) and their respective receptors, including the epidermal growth factor (EGF), sonic hedgehog (SHH), WNT, transforming growth factor (TGF-β), and Insulin-like growth factor (IGF-1) or Insulin. These growth factors can stimulate pancreatic cancer cell growth via autocrine and/or paracrine[1] feedback loops.

In this paper, we construct the first *in silico* model of the crosstalk between the six signaling pathways that have genetic alterations in 100% of pancreatic cancers, with the aim to investigate apoptosis, proliferation, and cell cycle arrest. Computational modeling and formal verification can help to better understand the interactions of multiple signaling pathways in the cancer cell. Given a model of a biological network, we are interested in verifying that sequences of signal activation will drive the network to a pre-specified state at or before a pre-specified time [2]. This can be achieved by Model Checking [3], an automated verification technique for finite state transition systems, which has been successfully applied for the verification of digital circuits and hardware protocols. Model checking is the process of determining whether or not a given model satisfies a desired property/specification written in a propositional temporal logic. Let M be a state-transition graph, S_0 be a set of starting states, ψ be a formula of temporal logic. The Model Checking problem is to verify that for all starting states $s \in S_0$, the model M satisfies ψ – written $M, s \models \psi$. Model checking algorithms exhaustively search the state space of the system model to determine the truth of specification. If the property is not satisfied, a counterexample will be given, *i.e.*, a sequence of transitions in the model M which starts from a state in S_0 and falsifies ϕ.

Several studies [2], [4], [5], [6], [7], [8] in systems biology have demonstrated that formal verification methods are a powerful alternative approach to study the dynamic behavior of biological networks. In this paper, we apply Symbolic Model Checking to study a number of important temporal logic properties in the proposed model of pancreatic cancer cell. Such properties have been verified by *in vitro* or *in vivo* experiments. We also propose several properties which could be tested by future experiments.

II. PANCREATIC CANCER CELL MODEL

Genomic analyses [1] have identified six cellular signaling pathways that are genetically altered in 100% of pancreatic cancers: the KRAS, Hedgehog, Wnt/Notch, Apoptosis, TGF-β, and regulation of G1/S phase transition signaling pathways. Also, many *in vitro* and *in vivo* experiments with pancreatic cancer cells have found that several growth factors and cytokines including IGF/Insulin, EGF, Hedgehog, WNT, Notch ligands, HMGB1, TGFβ, and oncoproteins including RAS, NFκB, and SMAD7 are overexpressed [9]. We performed an extensive literature search and constructed a signaling network model composed by the EGF-PI3K-P53,

[1] In autocrine signaling, a cell produces by itself growth factors to which it is sensitive. In paracrine signaling, growth factors are produced by a cell to stimulate another, nearby cell.

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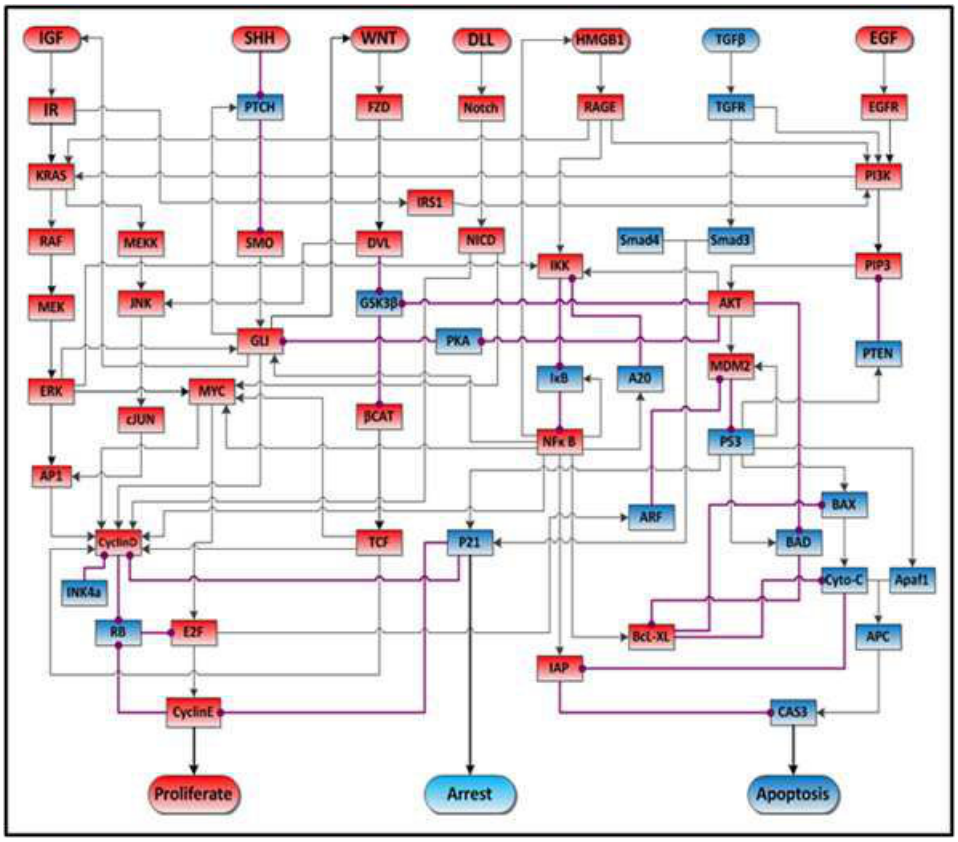

Fig. 1. Schematic view of signal transduction in the pancreatic cancer model. Blue nodes represent tumor-suppressor proteins, red nodes represent oncoproteins/lipids. Arrow represents protein activation, circle-headed arrow represents deactivation.

Insulin/IGF-KRAS-ERK, SHH-GLI, HMGB1-NFκB, RB-E2F, WNT-β-Catenin, Notch, TGFβ-SMAD, and Apoptosis pathway. Our aim is to study the interplay between tumor growth, cell cycle arrest, and apoptosis in the pancreatic cancer cell. In Fig. 1 we depict the crosstalk model of different signaling pathways in the pancreatic cancer cell. PI3K-P53, KRAS-ERK, RB-E2F, and NFκB pathways have been discussed in our recent work [2], [5], [6], [7]; here, we will briefly reiterate these pathways, and focus on the other genetically mutated pathways and their association with apoptosis, cell cycle arrest and tumor proliferation. In the following, the symbol $\rightarrow$ means activation (or overexpression), while the symbol $\dashv$ denotes inhibition (or deactivation).

Insulin/IGF-KRAS-ERK pathway: $Insulin/IGF \rightarrow IR \rightarrow KRAS \rightarrow RAF \rightarrow MEK \rightarrow ERK \rightarrow \{AP1, MYC\}$. The overexpressed growth factors, including Insulin-like growth factor (IGF) and Insulin, could activate the KRAS protein, resulting in the phosphorylation of its downstream proteins RAF, MEK, and ERK [10]. These can phosphorylate or activate the transcription factors AP1 and MYC to activate the expression of the cell cycle regulatory protein Cyclin D, enabling progression of the cell cycle through the G1 phase. KRAS is mutated in over 90% of pancreatic cancers [11]. This pathway could also upregulate the expression level of GLI in the sonic hedgehog pathway [1].

EGF-PI3K-P53 pathway: 1) $PI3K \rightarrow PIP3 \rightarrow AKT \rightarrow MDM2 \dashv P53 \rightarrow \{P21, BAX\}$; 2) $P53 \rightarrow PTEN \dashv PIP3 \rightarrow AKT \rightarrow MDM2 \dashv P53$. PDAC overexpresses a number of mitogenic growth factors and receptor tyrosine kinase (RTK), including EGF(R), IGF(R), which can activate the PI3K pathway to promote the growth of pancreatic cancer cells. The activation of PI3K initiates a cascade of reactions including the phosphorylation of PIP2, AKT and MDM2, leading to the inhibition of P53's transcription activity in the nucleus [12]. The tumor-suppressor protein P53, expressed in the later stage of PanIN, is mutated in more than 50% of pancreatic adenocarcinomas [11]. Also, P53 is a transcription factor for many tumor-suppressor proteins including PTEN and P21, which can negatively regulate the AKT pathway, and induce cell cycle arrest, respectively.

RB-E2F pathway: $CyclinD \dashv RB \dashv E2F \rightarrow CyclinE$. This pathway regulates the cell cycle progression from phase G1 to phase S, induced by the Cyclin E and CDK2 complex. In the normal cell, the unphosphorylated RB, a tumor suppressor protein, binds to E2F and inhibits its transcription activity. E2F will be activated when its inhibitor RB is phosphorylated and inhibited by Cyclin D, promoting the transcription of Cyclin E [13]. The germline mutations of CDKN2A in this pathway – which encodes the tumor suppressors INK4a (inhibitor of CyclinD-CDK4/6) and ARF (inhibit MDM2's activity to stabilize P53) – were found in up to 90% of pancreatic cancers [11].

SHH-GLI pathway: It is composed of two main parts: 1) $SHH \dashv PTCH \dashv SMO \rightarrow GLI \rightarrow$

$\{IGF, WNT, CyclinD, PTCH\}$; 2) $AKT \dashv PKA \dashv GLI$. The Sonic hedgehog (SHH) protein and its receptor Smoothened (SMO) are activated and overexpressed in later-stage pancreatic carcinomas, and it occurs in over 70% of PDACs [14]. In the quiescent cell without SHH, SMO is bound and inhibited by the tumor suppressor protein patched (PTCH). Once SHH binds to PTCH, SMO is released to activate the glioma-associated oncogene homologue (GLI1/2/3), leading to an active form of transcription factor. In the absence of SHH, the protein kinase A (PKA) and CKI (only PKA is shown in Fig. 1) transform GLI into a repressor form which can inhibit GLI's transcriptional activity. The activation of the SHH-GLI pathway is associated with tumor proliferation and pancreatic cancer-associated fibroblasts [15]. The expression of GLI could also be up-regulated by the PI3K-AKT and KRAS-ERK pathways, independently from SHH activation. In particular, SHH signaling alone is sufficient to drive pancreatic neoplasia, but does not form pancreatic adenocarcinomas [16].

WNT pathway: $WNT \rightarrow FZD \rightarrow DVL \dashv GSK3\beta \dashv \beta\text{-}Catenin \rightarrow TCF \rightarrow CyclinD$. WNT pathway activation and the overexpression of several pathway components were observed in 65% of pancreatic adenocarcinomas [17]. When the WNT protein is absent, β-catenin is localized in the cytoplasm, bound to and inhibited by the complexes composed of Axin, APC, and GSK3β [18]. The canonical WNT pathway is activated by the interaction of WNT and Frizzled (FZD) proteins, which can destabilize the Axin-APC-GSK3 complex and translocate β-catenin to the nucleus, where it activates the TCF-LEF transcription factors [19].

Notch pathway: $DLL \rightarrow Notch \rightarrow NICD \rightarrow CyclinD$. The Notch pathway is activated after binding of transmembrane ligands, including DLL (Delta-like 1, 3, 4) and Jagged 1-2 with Notch proteins. After that, Notch will be cleaved and a Notch intracellular domain (NICD) will be released, which will translocate to the nucleus to induce the expression of several target genes, including the cell regulatory protein Cyclin D. Recent findings indicate that the Notch pathway is involved in the development of pancreatic cancer [20].

HMGB1-NFκB pathway: $signaling \rightarrow IKK \dashv I\kappa B \dashv NF\kappa B \rightarrow \{A20, I\kappa B, BclXL, GLI\}$. A recent study [21] has found that the overexpression of HMGB1 could promote the growth of pancreatic cancer cells by activating the RAGE pathway. In the resting cell, NFκB is located in the cytoplasm, bound to and inhibited by IκB. Once activated by HMGB1, the IκB kinase (IKK) will phosphorylate and deactivate IκB, leading to the translocation of NFκB into the nucleus to promote the transcription of a number of genes, including Cyclin D, the anti-apoptotic protein Bcl-XL, its inhibitors A20 and IκB [22], [23], and HMGB1 [24].

TGFβ-SMAD pathway: It has two main parts: 1) $TGF\beta \rightarrow TGFR \rightarrow SMAD2/3/4 \rightarrow P21$; 2) $TGF\beta \rightarrow TGFR \rightarrow$ PI3K-RAS-pathway. The TGFβ-SMAD signaling pathway can inhibit the growth of normal human epithelial cells. When the TGFβ ligand binds to type II TGFβ receptors (TGFR), Type I receptors will be activated, leading to the phosphorylation of the cytoplasmic SMAD2/3 proteins. The proteins SMAD2/3 form a complex with SMAD4, and translocate into the nucleus to activate several transcription factors, upregulating the expression of cyclin-dependent kinase (CDK) inhibitors, including P21 [25], [26]. SMAD4 was found to be either mutated or deleted in over 50% of pancreatic cancers which occurred in the later-stage PanINs [27]. In addition to the Smad-dependent signaling pathway, TGF also activates the PI3K-RAS pathway, leading to the crosstalk with the WNT and EGF pathways. Impairment of the TGFβ-SMAD pathway promotes cell proliferation and contributes to carcinogenesis.

Apoptosis pathway: $P53 \rightarrow \{BAD, BAX, Apaf1\} \rightarrow cytochrome\text{-}C \rightarrow Cas3$. The apoptosis pathway is regulated by both the anti-apoptotic (BclX) and the pro-apoptotic Bcl-2 families of proteins [28]. The activation of P53 will induce or upregulate the transcription of several pro-apoptotic proteins including BAX, BAD, and Apaf1 (Apoptotic protease activating factor 1). After receiving pro-apoptotic signals from P53, BAD will inhibit Bcl-XL's pro-apoptotic effects, while this process is inhibited by the pro-survival signals from AKT. BAX is a protein of the Bcl-2 family which can activate the apoptosis process by promoting the release of cytochrome C (Cyto-C) from the mitochondrion. This, in turn, promotes the formation of the apoptosome complex (APC) [29] which contains Cyto-C and Apaf1. Cas3 is an apoptosis effector caspase (cysteine-dependent aspartate specific proteases) which can cleave proteins in the execution phase of cell apoptosis [30]. The activation of Cas3 is promoted by APC and inhibited by the inhibitors of apoptosis (IAP). It has been found that Cas3 is mutated in many cancer types [30].

III. Boolean Modeling

In this Section, we translate the above signaling pathways into a Boolean network model. The input signals of the model are different growth factors including SHH, EGF, TGF. The output signals are Apoptosis, (Cell) Proliferation, and (Cell Cycle) Arrest.

In the Boolean network model of the pancreatic cancer cell, each node represents a protein/lipid in the signaling pathway. At any specific time, each node can be in either the ON(1) or OFF(0) state. The state evolution of a node from time t to $t+1$ is described by a Boolean *transfer* function. This function will in general depend on the state of the neighbor nodes. In this paper we use several forms of transfer function. In one form, we assume that a node is activated (inhibited) if its incoming neighbor is active (inhibited). This form is used, for example, for receptor nodes such as EGFR, which are expressed only if their upstream ligand is present. A dual form assumes that a node is activated (inhibited) when its incoming neighbor is inhibited (activated). This form is used, *e.g.*, for SMO, which is bound and inhibited by PTCH (see the description of the SHH-GLI pathway above).

In another form, we assume that neighboring nodes are classified as *activators* or *inhibitors*. Activators node can change the state of a node n if and only if no inhibitor acting

on node n is in the ON state. Our assumption is motivated by the fact that many tumor-suppressor proteins including P53, PTEN, SMAD4, INK4a, and ARF, are either lost or mutated in the early or late stages of PDAC, while oncoproteins such as KRAS, NFκB, and GLI, are continuously activated or overexpressed. This convention has been successfully used in our recent HMGB1 Boolean network model [2], and in other works [31], [32]. The transfer function for node n can be written as

$$n(t+1) = \{n(t) \vee \bigvee_{a \in A(n)} a(t)\} \wedge \neg\big(\bigvee_{i \in I(n)} i(t)\big), \qquad (1)$$

where $A(n)$ and $I(n)$ are the activators and inhibitors of node n, respectively.

In our model, we assume *synchronous* state update for all the nodes in the network. That is, at any time step the state of each node in the model is updated according to its transfer function. Again, this assumption has worked well in our previous and others' works on Boolean modeling [2], [31], [32]. In the future we plan to study *asynchronous* models, to take into account the observation that biological processes may evolve at different speeds. We remark that our verification approach would still work, since Model Checking can cope with asynchronous systems.

The Boolean network in Fig. 1 comprises 61 nodes, including 7 control (input) nodes, and 3 output nodes. We emphasize that the structure depicted in Fig. 1 is not a state transition graph. Rather, it represents the "wiring diagram" of our model. Since each node is a Boolean variable, the state space of the model has cardinality 2^{61}. Is it a correct model to describe the proliferation and apoptosis of pancreatic cancer cell? To answer this question we use Model Checking of temporal logic properties, which we will introduce next.

IV. Symbolic Model Checking

A. Preliminaries

Let $AP = \{p, q, r, ...\}$ be a set of atomic propositions defined over a set finite set of states S. The Boolean logic connectives are: $\vee$ (or), $\wedge$ (and), $\neg$ (not), $\rightarrow$ (implies). Model Checking is an automatic verification technique for finite state transition systems modeled by Kripke structures. Let $S_0 \subseteq S$ be a set of initial states, $R \subseteq S \times S$ be a transition relation between states, and $L : S \rightarrow 2^{AP}$ be a function that labels each state s with the set of atomic propositions true in s. Formally, a *Kripke structure* is a tuple $M = (S, S_0, R, L)$ which represents a finite-state transition system. Given a Kripke structure M and a temporal logic formula ψ expressing some desired property about M, the Model Checking problem [3] is to find the set of all states in S that satisfy ψ, *i.e.* to compute the set $S_\psi = \{s \in S | M, s \models \psi\}$. If $S_0 \subseteq S_\psi$ we say that M satisfies ψ.

B. Temporal Logics

In this work we shall express our model's intended behavior as Computation Tree Logic (CTL) formulas. This logic has been developed to describe properties of computation trees, in which the root of the tree corresponds to an initial state and the other nodes on the tree correspond to all possible sequences of state transitions (paths) from the root [3]. A CTL formula is constructed from atomic propositions, Boolean logic connectives, *temporal* operators and *path* quantifiers. In particular, CTL has four temporal operators that describe properties of a path: $\mathbf{X}p$ – p holds in the ne**X**t state of the path; $\mathbf{F}p$ – p holds at some state in the **F**uture (eventually) on the path; $\mathbf{G}p$ – p holds **G**lobally (always) at every state on the path; $p\mathbf{U}q$ – p holds **U**ntil q holds. In a CTL formula, the operators $\mathbf{X}, \mathbf{F}, \mathbf{G}$, and $\mathbf{U}$ must be immediately preceded by a path quantifier $\mathbf{A}$ – for *all* paths, or $\mathbf{E}$ – there *exists* a path. For example, the formula $\mathbf{AG}(Shutd\_Req \rightarrow \mathbf{AX}Shutd\_Exe)$ means that whenever a *Shutdown Request* occurs, it will be always *Executed* in the next time step. It can be shown that any CTL formula can be expressed in terms of $\neg, \vee, \mathbf{EX}, \mathbf{EU}$ and $\mathbf{EG}$ [3].

CTL formulas can be divided into state formulas ψ and path formulas ϕ, and the syntax of the logic 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_0 is an initial state, $s_i \in S$, 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 state s_i. The semantics of CTL is defined as (the interested reader can find more details in [3]):

$M, s \models p$	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, $M, \pi \models \phi$

where $M, \pi \models \phi$ means that path π in M satisfies the path formula ϕ. Note that the temporal operators $\mathbf{F}$ and $\mathbf{G}$ can be defined as $\mathbf{F}\psi = true\,\mathbf{U}\,\psi$ and $\mathbf{G}\psi = \neg\mathbf{F}\neg\psi$.

C. Symbolic Model Checking

The Model Checking algorithm applied to a CTL formula ϕ works by recursively labeling the state graph with the sub-formulas of ϕ, and then parses the graph to compute, for each sub-formula, its truth value in a state according to the CTL operators and the truth values of its sub-formulas. In the original Model Checking algorithm, the state transitions were represented explicitly: this can lead to state explosion. To avoid this problem, in Symbolic Model Checking [33] the transition relation between states is represented implicitly using a Boolean function. In particular, this function is encoded by means of a Binary Decision Diagram (BDD) [34], a data structure that can be used to efficiently represent and manipulate Boolean functions. The Symbolic Model Verifier (SMV) [33] was the first CTL model checker based on BDDs. The NuSMV tool [35] is a reimplementation of the original SMV model checker. Using

the SMV language we can describe the models or state transition systems and verify the desired CTL properties. The output of the verification could be either "true" or a counterexample trace showing why the property is false. The interested reader can find the SMV code for our models at: http://www.cs.cmu.edu/∼haijung/research/PC.smv.

V. Model Verification

In this Section, we use Symbolic Model Checking to determine whether our *in silico* pancreatic cancer cell model satisfies certain properties written in a temporal logic.

In our model, we set the initial values of ARF, INK4a, and SMAD4 to be OFF (0), while Cyclin D is set to be ON (1). These choices are motivated by the following observations. According to the genetic progression model of pancreatic adenocarcinoma, the malignant transformation from normal duct to pancreatic adenocarcinomas requires multiple genetic alterations in the progressive stages of neoplastic growth, represented by Pancreatic intraepithelial neoplasias (PanINs)-1A/B, PanIN-2, PanIN-3 [11]. The loss of the functions of CDKN2A, which encodes two tumour suppressors - INK4A and ARF, occurs in 80–95% of sporadic pancreatic adenocarcinomas [36]. SMAD4 is a key component in the TGFβ pathway which can inhibit most normal epithelial cells' growth by blocking the G1-S phase transition in the cell cycle; and it is frequently lost or mutated in pancreatic adenocarcinoma [37]. Furthermore, it has been shown that the loss of SMAD4 can predict decreased survival in pancreatic adenocarcinoma [38]. Besides the loss of many tumor suppressors, the oncoprotein Cyclin D is frequently overexpressed in many human pancreatic endocrine tumors [39].

We divide the properties into three categories, according to their relationship with Cell Fate, Cell Cycle, and Oscillations.

Cell Fate:

The first properties we verify concern the pancreatic cancer cell's fate, *i.e.*, survival or death. In our model, the following two CTL properties are false:

$$\mathbf{AF}\,Apoptosis \qquad \mathbf{AF}\,Arrest$$

which mean that the cell does not *necessarily* have to undergo apoptosis, and that the cell cycle does not necessarily stop. On the other hand, the property

$$\mathbf{AF}\,Proliferate$$

is true, which means that the cancer cell will necessarily proliferate. Furthermore, since the following "steady state" property is true

$$\mathbf{AF}\,\mathbf{AG}\,Proliferate$$

we know that proliferation is eventually both unavoidable and permanent. We now ask whether it is always possible for the cancer cell to reach states in which Apoptosis and Arrest are OFF, thereby making cell proliferation possible. The following two properties are true:

$$\mathbf{AF}\,!Apoptosis \qquad \mathbf{AF}\,!Arrest\ .$$

However, the property

$$\mathbf{AF}\,(!Apoptosis\ \&\ !Arrest\ \&\ Proliferate)$$

is false, which means the model cannot always eventually reach a state in which apoptosis and cell cycle arrest are not inhibited and cell proliferation is active. We also report that the two properties

$$\mathbf{AF}\,\mathbf{AG}\,(!Apoptosis) \qquad \mathbf{AF}\,\mathbf{AG}\,(!Arrest)$$

are false, so that inhibition of apoptosis and cell cycle arrest are not unavoidable and permanent.

Cell Cycle:

We study properties involving the cell cycle, in which the protein Cyclin D is a key player. The next property is true:

$$\mathbf{A}(!Proliferate\ \mathbf{U}\ CyclinD)$$

which means that it is always the case that cell proliferation does not occur until Cyclin D is expressed (or activated). This property agrees with the experimental finding that Cyclin D is frequently overexpressed in pancreatic tumors [39]. However, in our model the activation of Cyclin D is not a steady state, since the following property is false:

$$\mathbf{AF}\ \ \mathbf{AG}\ CyclinD\ .$$

Next, we study the role of P53 in apoptosis. It is known that P53 can induce apoptosis through several signaling pathways [40]. Here, we ask whether in our model it is never the case that P53 is not activated until Apoptosis is activated. This question can be encoded in the following CTL formula, which is verified to be false:

$$!\mathbf{E}(!P53\ \mathbf{U}\ Apoptosis)\ .$$

Thus, Apoptosis can be activated even when P53 is not.

Oscillations:

There have been several experimental demonstrations of oscillations of NFκB signaling [23], [41]. We therefore ask whether our *in silico* model features oscillations, too. A CTL formula for encoding oscillations in NFκB is the following:

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

which turns out to be false. Next, we check whether overexpression of TGFβ can instead induce NFκB's oscillations. The formula

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

is in fact true, which means that an initial overexpression of TGFβ always leads to oscillations in NFκB's expression level. A similar property holds true for PIP3:

$$PIP3 \rightarrow \mathbf{AG}\,((!NF\kappa B \rightarrow \mathbf{AF}\,NF\kappa B)\&(NF\kappa B \rightarrow \mathbf{AF}\,!NF\kappa B))\ .$$

This property is actually an *invariant* of the model, since the following formula is also true:

$$\mathbf{AG}(PIP3 \rightarrow \mathbf{AG}\,((!NF\kappa B \rightarrow \mathbf{AF}\,NF\kappa B)\& (NF\kappa B \rightarrow \mathbf{AF}\,!NF\kappa B))).$$

It would be interesting to test experimentally the properties regarding TGFβ and PIP3.

Finally, oscillations have been detected in the expression level of P53 and MDM2. In Geva-Zatorsky *et al.*'s work [42], oscillations of P53 lasted more than 72 hours after cell damage induced by γ radiation. The next property is true:

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

which means that overexpression of P53 will always activate MDM2, which will in turn inhibit P53.

VI. Conclusions

We have presented and formally checked an *in silico* model for a single cell of pancreatic cancer. The model incorporates several important signaling pathways which are implicated with high frequency in pancreatic cancer. We have verified temporal logic properties encoding behavior related to cell fate, cell cycle, and oscillation of expression level in key proteins. The model agrees well qualitatively with experiments. We have also suggested several properties which could be tested by future experiments. Since verification is completed in a matter of minutes on a standard laptop, we conclude that Model Checking is a powerful approach for analyzing biological models.

VII. Acknowledgments

This work was supported by the NSF (award 0926181).

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