

The Evolution of Tumor Formation in Humans and Mice with Inherited Mutations in the p53 Gene

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Abstract While tumors are very heterogeneous in their origins, mutations in the p53 gene and inactivation of p53 gene functions are the most common feature that predispose to the formation of cancers in humans. Inherited p53 mutations lead to different tumor types at very different frequencies and at very different ages than somatic p53 mutations. The reasons for this are explored. When the first mutation arises in a stem cell (a gatekeeper mutation) it selects for a specific subset of second mutations which in turn select for mutations in a third subset of genes. The nature of the first mutation in a tumor determines, by selection, the functional types of subsequent mutations. Inherited mutations occur at different developmental times and in different orders of mutational sequences than somatic mutations. The excess risk of developing a cancer with an inherited p53 mutation is two- to three-fold in endodermal derived tissues compared with 100- to 1000-fold for ectodermal and mesenchymal derived tissues. By contrast, endodermal derived tumors with somatic p53 mutations occur at very high frequencies (70–100%). These evolutionary restrictions upon the mutational path that tumor development may take could open up new avenues for therapy and prevention.

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1 Background

In 1979, four research groups described a cellular tumor antigen, which was detected in viral, chemical and spontaneously transformed cells and came to be called the p53 protein (Linzer and Levine 1979; Lane and Crawford 1979; DeLeo et al. 1979; Kress et al. 1979). The p53 protein was shown to form a protein complex with the viral SV40 oncogene product, the large T-antigen (Linzer and Levine 1979; Lane and Crawford 1979; Kress et al. 1979) but was also shown to be found at high concentrations in chemically transformed cells (DeLeo et al. 1979) and in cell lines from testicular teratocarcinomas (Linzer and Levine 1979). Animals bearing tumors inoculated with these cell lines all produced tumors that produced antibodies directed against the p53 protein, classifying it as a tumor antigen. Subsequently, the p53 protein was found in a protein complex with the Adenovirus oncoprotein, the E1B 55Kd tumor antigen (Sarnow et al. 1982) and the Human Papilloma virus 12 and 18 E6 oncoprotein (Werness et al. 1990) demonstrating that several unrelated and diverse small DNA tumor viruses target this same p53 cellular protein by forming protein complexes. The Papilloma virus E6 protein was shown to aid in the polyubiquitination and ultimate degradation of the p53 protein (Scheffner et al. 1990) suggesting that the p53 protein was targeted by these viruses for inactivation or degradation. Oren and Levine (Oren and Levine 1981) first demonstrated that the levels of the p53 protein in non-transformed cells were regulated by a post-translational process producing a protein with a short half-life (degraded in 6–20 min).

Several groups were able to clone p53 c-DNAs from a diverse set of cell lines in culture. Two of these clones were shown to transform normal cells in culture (Eliyahu et al. 1984) while one of these clones not only failed to transform cells in culture but also prevented cells from being transformed by oncogene products (Finlay et al. 1989). The p53 c-DNA clones that transformed cells in culture were shown to arise from mutations in the p53 gene producing a protein that acted in a dominant negative fashion to transform cells (Hinds et al. 1989). The fact that the p53 gene functioned as a tumor suppressor gene preventing cancers was confirmed in cell lines (Wolf and Rotter 1984) and in human colorectal carcinomas where both alleles of this gene were found to harbor the same mutation (Nigro et al. 1989). Human families with germ line heterozygous mutations in the p53 gene were shown to develop tumors with a penetrance of about 93% (Hainaut and Pfeifer 2016) and knock out mice with a p53 gene deletion develop tumors with almost 100% penetrance (Donehower 1996) demonstrating that the p53 gene and its protein help to prevent tumors in mice and humans. The DNA sequencing projects of human tumors carried out over the past few years have demonstrated that p53 mutations are the single most common mutations found in human cancers suggesting a special role for the p53 protein in preventing cancers. There is a great diversity in the frequencies of p53 mutations in human cancers (serous ovarian cancers about 100% and testicular teratocarcinomas about 2%) suggesting that the cell type of origin and tissue type play an important role in p53 functions.

What are the functions of the p53 protein? It rapidly became clear that the p53 protein acted as a transcription factor binding to a specific set of DNA sequences to regulate gene transcription (Beckerman and Prives 2010; Zambetti et al. 1992; El-Deiry et al. 1992). Among the first set of genes shown to be regulated by the p53 protein were the p21 gene which blocks cell cycle progression in the G-1 phase of the cell cycle (El-Deiry et al. 1992) and the MDM-2 gene (Momand et al. 1992) which is an E-3 ubiquitin ligase that polyubiquitinates the p53 protein and leads to its degradation. The crystal structure of the p53–MDM-2 complex was elucidated (Kussie et al. 1996) and this led to the development of a class of drugs, called the nutlins (Vu et al. 2013) that disrupt the p53–MDM-2 protein complex and this activates the wild type p53 protein which causes a reduction in tumor growth by killing the cancer cells. The fact that the p53 protein promotes the transcription of the MDM-2 gene while MDM-2 leads to the degradation of the p53 protein, creates an auto-regulatory feedback loop (Wu et al. 1993) and the oscillation of these two proteins 180 degrees out of phase in the cell (Bar-Or et al. 2000; Lahav et al. 2004). As the number of diverse genes regulated by the p53 transcription factor became clear (Riley et al. 2008) several transcriptional programs that enhance tumor suppression were uncovered. The activation of the p53 protein, so that it functions as a transcription factor, could lead to apoptosis or cellular senescence killing the cell before it can become cancerous. p53 was shown to mediate G-1 and G-2 cell cycle arrest. p53 regulated the number of centrosomes produced during the cell cycle. It had a large impact upon the metabolic profiles used by cells regulating the insulin-like growth factor pathway, mitochondrial functions, glutathione production and shifts between the Warburg effect and normal oxidative phosphorylation. p53 regulated cell mobility, invasiveness and wound healing. The p53 protein was shown to regulate many genes involved in DNA repair processes and epigenetic changes in cells. Each of these diverse transcriptional programs is regulated by an activated p53 protein that is modified by a variety of enzyme activities that are also employed to modify histones forming the chromatin template for transcription. These p53 protein modifications (phosphorylation, methylation, acetylation, ubiquitination, etc.), arise in a cell undergoing one or more types of stress.

A wide variety of DNA damaging agents promote p53 activation, and an increase in p53 levels. The high levels and protein modifications result in p53 dependent transcriptional programs resulting in the transcription of genes whose products are involved in DNA repair and/or cell death (Maltzman and Czyzyk 1984; Kastan et al. 1991). These two publications by Maltzman in 1984 (Maltzman and Czyzyk 1984) and Kastan in 1991 (Kastan et al. 1991) demonstrate a common issue in scientific research; an important discovery made seven years before and forgotten by the field is rediscovered and becomes a central core in our understanding of how cancers are prevented by the p53 protein. Maltzman was trained at Stanford in a laboratory that worked on DNA repair processes. He carried out his postdoctoral work at Princeton working on the p53 protein in the Levine laboratory and then took a position at Rutgers. There he met Evelyn Witkin who had discovered the rec A gene central in controlling cell division in *E. coli* and responding to DNA strand breaks. Witkin was reading a paper about the p53 protein and its

fluctuations in the cell cycle (Reich and Levine 1984) and saw real similarities between Rec A functions and p53 functions and she told Maltzman about that. Maltzman (Maltzman and Czyzyk 1984) (in 1984) then carried out the experiments that demonstrated that the p53 protein responds to DNA damage by ultraviolet exposure increasing its concentration (no longer being regulated by MDM-2 which would be found eight years later), acquiring post-translational modifications, and transcribing a set of genes to kill the cell (which would be uncovered ten years later). No one followed up these experiments until Kastan rediscovered that p53 responds to DNA damage in 1991. At that point, the p53 field was ready and realized that the p53 protein functioned to respond to stresses like DNA damage.

Today, a large number of cellular stresses, in addition to DNA damage, are known to activate the p53 protein and initiate a transcriptional program that responds to stress. Hypoxia, nutrient deprivation, telomere shortening, oncogene activation by Ras, myc or viral oncogenes, epigenetic reprogramming of cells and virus infections, cytokine exposures and failures in ribosomal biogenesis are all activators of a vigorous p53 response. Just which transcriptional program is then carried out by p53 to repair or kill the cell depends upon a large number of variables: the cell and tissue type, whether a cell is transformed by an oncogene or not, the chromosomal ploidy of a cell, the stage of differentiation of a cell (stem cell, progenitor cell, differentiated cell) and the lineage of a cell's differentiation program. The p53 transcriptional program can synthesize cytokines or interferons and call in the immune system to clear out dead cells and present neo-antigens to the immune response. A role for p53 in the central nervous system where it can sense DNA damage and respond with cell death and neurodegeneration is a viable hypothesis that needs to be tested in more detail. A role for p53 in sensing and responding to the microbiome is also suggested by some observations and now requires a set of follow-up experiments. The p53 protein is known to monitor ribosomal biogenesis making sure cells will produce enough ribosomes for cell division to proceed. Inherited mutations in some ribosomal protein genes give rise to defects in an optimal rate of ribosomal biogenesis and activation of p53, which can result in Diamond Blackfan Anemia in humans do to inhibition of cell cycle progression and death of reticulocytes during red blood cell production in the bone marrow. Clearly, the p53 tumor suppressor is a double edge sword both protecting against errors during cell division and preventing cancers from arising but also responding to stresses by killing essential cells that can then compromise normal physiology.

2 The Li-Fraumeni Syndrome

The Li-Fraumeni Syndrome (Oliveri et al. 2010) is an autosomal dominant disorder that results in human families that develop a characteristic subset of tumors commonly at a young age. About 70% of these families harbor p53 mutations in the heterozygous state, which reduce to homozygosity at a high rate and begin a

Table 1 The impact of the germ line cell types upon the excess risk of developing a tumor with inherited p53 mutations or the frequency of somatic p53 mutations in tumors that arise over a lifetime

Germ line contribution	Inherited excess risk	Somatic mutation frequency (%)
Ectoderm	10–1000×	2–35
Mesoderm	10–100×	50–60
Endoderm	2–3×	70–100

process, which results in one or more cancers appearing over a lifetime with a penetrance of 93%. The International Association of Cancer Research (IARC, Lyon, France) collects and stores a great deal of information about these families and the analysis described herein was carried out by Pierre Hainaut and is based upon the R16 version of the IARC database (November, 2012). These data sets describe 634 families with p53 germ line mutations producing 2483 cancer cases with a 30,000 person-year follow-up of these individuals. One of the surprising observations made with these families was that individuals who inherit a p53 mutation develop cancers during their lifetime that derive from an ectodermal and mesenchymal lineage with a ten- to one-thousand-fold excess risk than are observed in wild type p53 populations, while the excess risk of developing a tumor derived from the endodermal lineage is only two- to three-fold higher than the wild type population (Table 1).

This is in contrast to tumors with spontaneous somatic p53 mutations acquired over a lifetime in endodermal tumors of the colon (75% mutations), lung (75% p53 mutations), pancreas (80% p53 mutations), and serous ovarian tumors (100% p53 mutations). Clearly, there is a remarkable difference in the frequencies of p53 mutations that cause endodermal tumor types produced by inherited p53 mutations and spontaneous p53 somatic mutations and the reasons for this remain unclear. Note, however, that inherited p53 mutations position the p53 mutation to be the first mutation in a series of mutations that cause the cancer. Thus, the p53 mutation can be classified as the gatekeeper mutation and this modifies the cell so that additional mutations in other genes can be selected for that contribute to cell growth and division. Spontaneous p53 mutations that occur in somatic tissues over a lifetime are not commonly the gatekeeper or first mutation in a series, so the selection for the next set of mutations in progressing to a tumor alters the evolution of that tumor. This explanation suggests that the order of mutations in the formation of a tumor determines the subsequent genes selected for based upon the nature of the gatekeeper mutation. There are a growing number of studies that support this hypothesis.

In mice that have inherited p53 mutations in both alleles, the next mutation is always a PTEN mutation, followed by a cdk6 and cyclin D amplification and finally a notch pathway defect (Fig. 1) (Dudgeon et al. 2014).

Cells carrying a p53 mutation are more susceptible to promoting genomic instability. If the rare cell that has p53 mediated genomic instability acquires a

P53	→	PTEN	→	Cycle D Cdk-6	→	NOTCH Pathway
-/-		-/-		Amplified		Splicing Alterations

Fig. 1 The order of selection of mutations in thymic lymphomas from ten different $p53^{-/-}$ mice, the $p53$ mutation, $-/-$, was inherited, the $PTEN$ deletions arose before the rearrangement of the T-cell receptors in the progenitor T cells. Gene amplifications were then observed in cyclin D1, 2 or 3 genes and the $cdk6$ gene followed by mutations in the notch pathway. The $p53$ mutations give rise to genomic instability. The $PTEN$ mutations give rise to the high utilization of glucose and metabolic alterations permitting rapid cell division. The cyclin D- $cdk6$ mutations accelerate the cell cycle times and the notch mutations block T cell development and differentiation keeping the tumor stem cells progenitor cells

homozygous $PTEN$ mutation, it provides higher glucose levels for the cell to alter its metabolic program so as to support enhanced cell division, increasing the clone size. This is followed by enhancing the rate limiting steps in the G-1 phase of the cell cycle, cyclin D- $cdk4/cdk6$ phosphorylation of Rb. In $p53^{-/-}$ cells, this is accomplished by gene amplifications of any one of the three cyclin D-1, 2 or 3 genes and the gene for $cdk6$. By shortening the G-1 phase of the cell cycle and constantly bring cells back into cell cycle while the need for high levels of glucose is maintained, one continues the clonal expansion of the precursor to a thymic lymphoma (Dudgeon et al. 2014). Finally, mutations in the notch pathway restrict the T-cells from fully differentiating keeping a progenitor cell in division (the tumor is a CD-4+/CD-8+ double positive T-cell lymphoma). Each of these four steps in the development of the tumor is ordered and contribute to different cellular processes: 1. Genomic instability and loss of apoptosis, 2. An increased ability to take up glucose and to employ Warburg aerobic glycolysis to supply both energy and substrates for growth, 3. Enhanced rates of cell division by reducing the rate limiting aspects of the G-1 phase of the cell cycle and 4. Eliminating the final steps in differentiation of T cells giving rise to a thymic lymphoma.

In colon cancers, the most common gatekeeper mutation is in the Wnt pathway (APC mutation or beta catenin mutation), which is followed by a Ras mutation, then a TGF-beta mutation and finally a $p53$ mutation (Fearon and Vogelstein 1990). That the order of these mutations was critical to the development of colon cancers was demonstrated in mice (Takeda et al. 2015). Insertional mutagenesis was employed to promote colon cancers in mice that inherited (the gatekeeper mutation) an APC mutation, a Ras mutation, a TGF-beta mutation or a $p53$ mutation. Tumors arose fastest in time in the genetic background that carried the APC mutation. They arose second in time in the RAS-mutated background, third in time in the TGF-beta background and it took the longest time to observe tumors of the colon in mice with a mutation in the $p53$ gene. Since these mutations likely occur spontaneously in

populations that do not start with a gatekeeper mutation and therefore occur stochastically over time and cell divisions, the simplest explanation for these observations is that the order of mutations results from the selection of the next mutation in cells that divide and survive so as to expand the lineage ultimately leading to a rapidly growing and dividing cancer. In other words, a Wnt pathway mutation next selects for a Ras mutation to get the cells into cycle, followed by the loss of the negative regulator of growth and division, TGF-beta. All of these mutations may happen in the stem cell of the colon keeping the benign tumor or polyp in situ or in the crypt. A final p53 mutation enhances cell migration, invasion and penetration of the colon wall, giving rise to a colon or colorectal carcinoma. This line of reasoning does not eliminate the possibility that the first or previous mutation results in the enhancement of the mutation rate in a cell, resulting in an optimal order of mutations that give rise to a tumor rapidly. These ideas do help to explain why one can find tumor suppressor gene mutations and oncogene cancer driver mutations in cells in the body that have not given rise to a cancer and must be awaiting a mutation in a gene that expands the clone and results in a malignant cancer. The fact that many stem cells (colon, skin, pancreas, breast, etc.) rely upon stem cells that are driven by the Wnt pathway predicts that Wnt pathway mutations might well be a common gatekeeper mutation.

A second interesting observation that derives from the Li-Fraumeni data set is that the age range at which different tumor types occur and are diagnosed in a Li-Fraumeni patient are limited to specific times during a life span and are reproducible in many different patients. For example, adrenalcorticocarcinomas (ADC) occur between the ages of 6 months to 4 years; choroid plexus papillomas (CPT) between 6 months and 3 years, medullary blastomas (MED) between 2 and 9 years and Rhabdomyosarcomas (RMS) between 1 and 4 years of age, all at 50- to 1000-fold higher excess risk than observed in the wild type population (Table 2).

Liposarcomas (LPS) and osteogenic sarcomas (OST) commonly occur from 1 to 15 to 20 years of life with a ten-fold excess risk compared to the wild type population. Breast cancers (BC) occur in 50% of the Li-Fraumeni females between

Table 2 The ages of diagnosis and excess risk of different tissue types of inherited p53 mutations ADC, Adrenalcorticocarcinomas; CPT, Choroid Plexus Tumors; MED, Medulloblastomas; RMS, Rhabdomyosarcomas; LPS, Liposarcomas; OST, Osteogenic sarcomas; BC, Breast Cancers, LMS, Leiomyosarcomas

Tumor type	Ages	Excess risk
ADC	6 months–4 years	100–1000×
CPT	6 months–3 years	100–1000×
MED	2–9 years	100–1000×
RMS	1–4 years	100–1000×
LPS, OST	1–20 years	10–1000×
BC	18–45 years	50% of females
LMS	20–55 years	10–100×

18 and 45 years of age and leiomyosarcomas (LMS) and liposarcomas (LPS) occur about 20–55 years of age (Table 2). In other words, in patients with Li-Fraumeni syndrome and heterozygous for a p53 mutation, tumors of specific tissue types occur at specified times or ages during a life span. This suggests that p53 loss and functions act differently in different tissues. Alternatively, p53 functions could be most important in actively growing tissues or stem or progenitor cells which are determined by developmental programs active at different times during the life span of a person.

Interestingly, the excess risk of developing a first cancer in an individual with a heterozygous p53 mutation falls below 1.0, or lower than the wild type population, when the individual with a p53 mutation is over the age of 65–70 years. This of course is a time when the wild type population is increasingly at risk for developing cancers. So why would a person at age 75 or 80 years of life with a p53 mutation that failed to promote a detectable cancer over a lifetime actually have a lower risk of developing a cancer than a person born with two wild type alleles in the p53 gene? This observation may be ascribed to good luck, remaining in a good environment with a low mutation rate over a lifetime or the presence of genetic suppressors in an individual that are able to compensate for the presence of only one copy of a wild type p53 gene over a lifetime. The fact that enzymatic activities, like histone acetyltransferases or methylases, that modify the p53 protein can impact its activity and specificity as a transcription factor, suggest one class of p53 modifiers, activators or suppressor polymorphisms that may increase the levels or activity of a p53 protein that is made from a single wild type copy of DNA so that it would be equal or better than two copies of wild type p53 in a person. Mutations or polymorphisms in enzymatic activities that alter the epigenetic program might also result in greater longevity, a healthier life at older ages and resistance to the development of cancers. This provides a hypothesis that is testable in the 7% of individuals that have p53 mutations, with Li-Fraumeni syndrome, but fail to develop cancers over their lifetime. It may also be testable in centenarians that are in good health and have never developed a cancer.

Individuals that have inherited p53 mutations and develop cancers throughout their lifetimes are at risk for different tumor types at different ages of developmental time (Table 2). An examination of the tissue types susceptible to developing cancers in Li-Fraumeni patients with a p53[±] genotype occurs during the time of life with very active cell divisions and the activities of tissue-specific stem cells in the mesenchymal and ectodermal lineages, but not the endodermal lineage. These data link p53 functions to selected stem cell populations derived from ectodermal and mesenchymal lineages during their most active rates of cell growth and division. Here, p53 functions as a tumor suppressor by eliminating actively dividing cells that undergo stresses that create high error rates in DNA sequences, processes of cell division, faithful segregation of chromosomes or even undergo abnormal epigenetic alterations.

3 Li-Fraumeni Syndrome in Mice

The observations made in humans with a $p53^{\pm}$ genotype provide interesting associations and correlations, but the diverse genetic backgrounds of humans and different environmental exposures over a lifetime introduce quite a few variables that can influence the results and interpretations of these data sets. Even the rare occurrence of identical twins with $p53^{\pm}$ genotypes that are brought up in the same family and environment provide limited examples and environmental differences that are difficult to control. For example, a Li-Fraumeni family in Malaysia had a pair of identical twins who were confirmed to both have the same $p53$ mutation and almost identical DNA sequences (there are about sixty differences in nucleotide sequences mostly in non-coding regions) and were brought up in similar environments in the same family. One twin developed independent tumors at 4, 9 and 21 years of age while the other twin has not developed any tumors as of today. This difference could be an example of an environmental triggering event (exposure to X-ray DNA damage, etc.) in only one of the twins but this is not recorded in the medical records. At the minimum, we can conclude that random or stochastic events could be a determining factor in tumor initiation.

One solution to the limited number of identical twins with Li-Fraumeni Syndrome in humans is to create mice with $p53^{-/-}$ genotypes and \pm genotypes from an inbred strain and who share the same cages in an animal colony over a lifetime. We can then record the frequencies of tumors, tumor types that develop and the ages of onset of the tumors. This type of study produces better statistics, permits one to vary the genetic background and even the environment such as the exposure to radiation. $p53^{-/-}$ mice of many inbred stains develop thymic lymphomas as a tumor early in life and if they survive they can develop tumors of the mesenchymal and ectodermal lineages later in life. Thymic lymphomas are never or rarely observed in human Li-Fraumeni Syndrome patients. $p53$ heterozygous, $p53^{\pm}$ mice develop tumors later in life with more mesenchymal and ectodermal tumors observed and fewer thymic lymphomas. If these mice are irradiated with X-rays, then tumors develop earlier in life and both leukemias, lymphomas and solid tumors arise.

A $p53$ male mouse homozygous for the $p53$ 172 mutation in the C57 Bl/6 genetic background was mated to $p53^{+/+}$ females from eight different genetic backgrounds, and the type of tumors that were formed in the F-1 hybrid offspring mice were determined at autopsy by histological study analyzed by a pathologist. This experiment was carried out to determine whether different inbred stains of mice carried dominant (in the F-1 offspring) suppressors or modifiers of the tissue types that would form tumors in a $p53^{\pm}$ mouse. Does the genetic background of the host help to determine or modify the probability of tumor formation due to loss of the $p53$ gene function? Table 3 presents the results of this experiment. Based upon this analysis, it is quite clear that the genetic background of the host can influence the frequencies and types of tumors that arise in cells with a $p53$ deficiency.

Table 3 Tumor tissue types in p53[±] F-1 mice from eight different genetic backgrounds p53 wild type mice from the genetic background indicated in the strain column were crossed with C57Bl/6 mice of the p53^{-/-} phenotype producing p53[±] F-1 mice. The frequency of tissue types of tumors that arose over one and a half years are indicated

Strain	Lymphoid Hyperplasia Lymphoma	Osteosarcoma	Adenocarcinoma	Spindle Cell Sarcoma	Angioma/Angiosarcoma	Squamous Carcinoma	Hemangioma	Lipoma Lipomatous Tumor/Liposarcoma	Totals
129sl	5	3	1		1	1	1	1	13
A/J		5	1	2	1			5	14
BalbC	2	5	6	2	7	3	1		26
C3H	6	2	1	1					10
C57	8	1	4	2	1		2		18
DBA	6	1	2		1		1	1	12
NOD	18	12	2	6	1	1	2		42
SWR	21	14	8	3	3	6	1		56
Total Cases	66	43	25	16	15	11	8	7	191

The great majority of tumors have lost the wild type copy of the p53 gene in the tumor but occasionally the tumors are heterozygous for the p53 gene. There are some genetic backgrounds (C3H, C57Bl/6, DBA) where 50–60% of the mice develop thymic lymphomas, some (NOD, SWR) where 40–43% of the mice develop thymic lymphomas and others like AJ mice had no thymic lymphomas (out of 14 tumors produced) or BALB/c mice had only 4% thymic lymphomas (2/26 tumors produced). 5/7 liposarcomas produced in 191 tumors observed in these mice from diverse genetic backgrounds were produced in the AJ mice. 7/15 angiosarcomas were made in the BALB/c F-1 mice. Mice with diverse genetic backgrounds have different predispositions to develop different tumor types, and the loss of the p53 tumor suppressor gene appears to exaggerate these predispositions. In all of these mice, 66/191 tumors were thymic lymphomas (34% of the tumors formed), and this type of tumor is not observed in humans with a p53[±] genotype. Clearly, the species under study introduces differences we need to understand if we are to use these animals as models for the human disease.

A number of these tumors have had their DNA sequenced so that one can ask whether all thymic lymphomas have the same genes mutated when p53 mutations act as the gatekeeper independently of the genetic background or the age of the animal when the tumor developed? In p53^{-/-} mice in the C57Bl/6 genetic background, all the thymic lymphomas harbored a common set of mutations (Dudgeon et al. 2014). The p53 mutation is inherited. The next mutation selected for is a homozygous loss of the PTEN gene. That is followed by gene amplifications in the cyclin D genes and the cdk6 gene or alternatively the deletion of Rb (retinoblastoma gene), which is the substrate for the cyclin D-cdk6 kinase. Finally, mutations in the notch pathway are found that compromise the differentiation of the T cells that form the lymphoma (Table 2). In the SWRx C57Bl/6 F-1 mice with a p53[±] genotype, thymic lymphomas can be detected as early as 4.7 months after birth or as late as 18.5 months after birth. We are sequencing these early and late thymic lymphomas to determine if the differences in the time it took for the formation of a tumor result in differences in the genes that contribute to the formation of that tumor. Similarly, we observe osteogenic sarcomas arising in SWRx C57Bl/6 F-1 mice at 11 months of age and at 19.6 months of age. We are sequencing these two tumors to examine the variables of age of tumor formation, mutation frequencies, and accumulation over a life span. These experiments permit us to study the nature of the driver mutations in tumors with diverse incubation periods in mice that are genetically identical and environmentally constant and when the tumor tissue type producing a tumor is identical.

4 Quantitation of the Clonal Evolution of Thymic Lymphomas in Mice with Mutant p53 Proteins

The thymic lymphomas produced in p53^{-/-} mice in the C57Bl/6 genetic background permit us to ask a specific set of questions about the cellular clonal evolution of these tumors. During fetal life, a precursor to the T-cell lineage migrates to the thymus

where the T cells will produce unique receptors by both recombination of the V-D-J genes and the insertion of nucleotides by terminal transferase to produce four different chains of the T-cell receptor dimer. Each T cell that is produced has a distinct T-cell receptor amino acid sequence in the hyper-variable region of the gene and protein. This region at the DNA level spans about 200 nucleotides in the DNA copy of a T-cell receptor and it has been possible to set PCR primers on either side of this DNA region, amplify the sequences from millions of T-cell receptors produced in a thymus in a quantitative and reproducible fashion and sequence these receptors (Dudgeon et al. 2014). This permits us to ask a number of questions: Are the thymic lymphomas that are formed several weeks after the birth of a mouse clonal, that is derived from a cell with a single T-cell receptor sequence, or are they oligoclonal? If they are oligoclonal tumors what is the frequency of transformation in the thymus? How many new cancer cells arise per day or week? Do different clones that arise then compete so that an oligoclonal tumor early in life resolves itself into a dominant clone later in life, which then kills the host? What are the nature of the additional mutations that permit some malignant cell clones to be the most fit for survival? By sequencing the DNA from heterogeneous or homogeneous tumors can we determine a frequency of point mutations in the genome? Can we determine the frequency of copy number variations or gene amplifications, deletions, inversions, insertions, aneuploidy or other types of mutations that are mediated by the absence of the p53 gene and protein? By carrying out these experiments (Dudgeon et al. 2014), it has been possible to answer some of these questions. First, mice that are born with p53 mutations in both alleles do not have evidence of a thymic lymphoma. p53 is necessary but not sufficient for the production of these tumors. By nine weeks of life, the first indication of thymic lymphomas is observed, so that additional mutations are required to produce a tumor when p53 mutations are the gatekeeper. At nine weeks, the thymic lymphomas are oligoclonal, with 2–10 different T-cell receptors being detected in a thymus. The clones continue to be produced with new receptors (de novo) and some existing clones continue to expand becoming dominant clones in the thymus and other lymphoid organs (up to 70–90% of the tumor). Between nine weeks of life as malignant clones arise and twenty weeks of life as some mice die of their disease, new clones continue to arise in the thymus at an average frequency of 0.13–0.80 clones per day (from nine weeks to twenty weeks) with that much variation between different mice of the same genetic background and the same gatekeeper mutation (Table 4).

Sequencing the DNA from these thymic lymphomas at 20 weeks (70–90% homogeneous tumors) demonstrates a point mutation frequency of single nucleotides of one per megabase. This is not extraordinarily high, being a similar frequency for human breast or pancreatic cancers. However, aneuploidy is very common; with 4–8 chromosomes per tumor with one or more aneuploidy chromosomes (chromosome 5) reproducible between tumors from different mice. There were also a large number of copy number variations (276–422 per tumor) producing deletions, amplifications, inversions, recombinations in every tumor. Up to 175 different genes or regions of chromosomes were commonly amplified in most tumors (Table 4). Chromothripsis, where a chromosome is broken into many parts

Table 4 The clonal evolution of thymic lymphomas in $p53^{-/-}$ mice, the DNA exons from three independent thymic lymphomas that arose in three different $p53^{-/-}$ mice were sequenced giving a frequency of point mutations per megabase and the number of copy number variations (CNV) per genome in the tumors. The T-cell receptors from these tumors were sequenced and the number of abnormally high levels of clonal T-cell receptors (tumor cells) detected in the tumor indicated the number of independently transformed cells that produce a tumor over the period of 9–20 weeks expressed as the transformation frequency per day. These data are from 20 different tumors

1. Frequency of point mutations	1/megabase
2. Number of CNV	276–422/genome
3. Number of transformed clones (9–20 weeks)	0.13–0.80/day

and reassembled in a more random order, was fairly common in $p53$ mutant cells of these tumors. It appears that the absence of $p53$ as a gatekeeper mutation permits a much higher frequency of copy number variations in the genome and most of the losses of tumor suppression genes arise from deletions and most of the oncogene activations occur from gene amplifications and aneuploidy. In this case, $p53$ is correctly named “the guardian of the genome.”

5 Conclusions

Evidence is presented that cancers arise from the presence of a first or gatekeeper mutation in a stem cell or progenitor cell. The nature and functions of a gatekeeper mutation then determine the order of subsequent mutations, which are selected for by a clonal expansion. Each subsequent mutation only functions to contribute to the formation of a cancer when it is selected for by the functions of the previous mutations. Mutations may well arise in a random fashion but are only selected for by the properties of a cell that will enhance its fitness from that mutation. This helps to explain why inherited mutations in tumor suppressor genes (where they are gatekeepers) produce tumors of different tissue types, then when the same mutation in the same gene acts as a somatic mutation later in life (not a gatekeeper mutation). The order of a set of mutations plays a critical role in the tissue type, outcome and evolution of a cancer. This also helps to explain the ever more common observation that mutations in cancer driver genes are detected (by sequencing or single cell sequencing) in stem cells or progenitor cells of people that do not have a cancer. Such mutations are predispositions in the sense they are waiting to be paired with a gatekeeper mutation that permits them to be selected for in the population.

This hypothesis would seem to predict that all tumors of a particular tissue tumor type would follow a single order of selected mutations and produce homogeneous tumors, when in fact we are finding great heterogeneity between tumors of the same tissue types. This can largely be understood by the hundreds of additional and unique mutations that arise in these tumors (genomic instability, epigenomic instability). They may contribute to invasiveness, metastasis, metabolic differences,

altered mutation rate, altered DNA repair, drug resistance, etc. These mutations may arise during the competition for fitness of clones that are produced by the tumor cells. But the take home message for successful treatment is our focus upon inhibiting or activating those gatekeepers and subsequently selected mutant genes that are fundamental drivers of the tumor. BRAF mutations alone produce a nevus or benign tumor. In a melanoma, BRAF mutations can be inhibited and contribute to remission of the tumor, but resistance arises and the tumor proceeds. If it is correct that most stem cells utilize the Wnt pathway for driving cell growth and creating a pool of stem cells to produce progenitor cells and stem cells are the place where gatekeeper mutations become established, then inhibitors of the Wnt pathway might be the best drugs to treat cancers. However, we need to understand how to block a Wnt pathway gene without stopping normal stem cell replication. We rely upon these stem cells for regenerating ourselves throughout life. Perhaps we can develop drugs that block cell division or promote cell death in a cell that has two mutations like p53 and PTEN (thymic lymphomas) or Wnt and RAS (colon cancers). We clearly need to better understand the fundamentals of the origins and evolution of cancers, and we need real novelty in planning the path to treatments and prevention of cancers.

As part of this challenge to the field, we have a real clue that must be followed. The single most common mutation in human cancers occurs in the p53 gene. These mutations are most often missense mutations largely localized to the DNA binding domain of this transcription factor. There are more than 220 different mutant alleles but each when measured has a similar defect, the protein only binds poorly to the DNA sequence that the wild type p53 protein binds to and each allele tested is a poorer transcription factor for a set of p53 regulated genes. These mutations are therefore loss of function mutations. Can we restore their functions or find synthetic lethal gene functions that kill cells with a p53 mutation? Many of these missense mutant p53 proteins appear to bind to other cellular proteins, and there is increasingly convincing evidence that these protein–protein interactions contribute to several gain of function phenotypes that promote growth, alter metabolism, add to drug resistance, enhance tumorigenic potential, enhance invasiveness of cancer cells and may alter the tumor types observed with different alleles of p53 mutations. If this is correct, will it be possible to develop drugs that eliminate the gain of function phenotype? Will this be a useful therapeutic approach? One would think that such a drug may have only limited or no side effects if it indeed acted to inhibit a mutant gain of function, what normal cellular function would it act upon?

Finally, if we had an efficient way to introduce the wild type p53 gene or c-DNA into all of the cancer cells in a patient, we could then kill these cells. To accomplish this, we require two new technologies we do not have. We require a delivery system (viral gene therapy, DNA or RNA, reagent or chemical) that targets cancer cells and not normal cells, and we require a targeting therapy that is efficient enough to kill all the cancer cells in the body. While to date this has not been a very productive path, there are some new ideas or approaches that should be explored. Can we activate and organize the immune system to work on these questions that require this type of specificity and selectivity and efficiency, three interesting properties of the immune

system. Can we modify immune cells to recognize cancer cells but not normal cells? Could immune cells or products of immune cells be used to enhance specificity and the efficiency of killing tumor cells? Alternatively, is there a role for the microbiome to communicate with the immune system leading to tumor-specific cell killing, activation of p53 in a cancer cell or introducing wild type p53 c-DNAs? These kinds of ideas are likely to be funded and explored by private foundations that are taking a leading role in the exploration of ideas that might lead us to new technologies that in turn will move the field in new directions. This is basic science and this is needed.

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A Personal Note As an undergraduate at The State University of New York at Binghamton, Harpur College, I took a course in microbiology and became fascinated with viruses. Viruses were too small to observe in a light microscope, were obligate intracellular parasites and yet reproduced themselves in an infected cell. How much genetic information did it take to reproduce oneself? Are viruses the smallest and simplest form of life? If so, would the study of viruses tell us what it was about life, life processes and reproduction of self that would lead us to the fundamental principles of biology. In the 1960s, I was a graduate student at The University of Pennsylvania and a postdoctoral fellow at The California Institute of Technology and worked with viruses (adenoviruses, SV40 and bacteriophages). That is when I learned it might well take a lifetime to answer some of these questions. I also was exposed to many scientists in the literature, at meetings and in courses taught at Cold Spring Harbor, Aspen Colorado, Woods Hole Massachusetts, and Bar Harbor Maine. The 1960s were a time for virus isolation, classification, uncovering the styles of virus replication and the role of the host cell. Among the leaders in describing these events in the viruses of chickens was Peter Vogt. His clarity, organization, attention to detail and insights set the standard for the field. By the 1970s, he laid the groundwork and actively contributed to the major events and publications that changed the field, providing the evidence that RNA tumor viruses, the retroviruses, contained oncogenes that were the mutant forms of cellular genes. By contrast, the DNA tumor viruses that I worked with contained their own oncogenes. Why? While the paths of the RNA and DNA virologists began to separate, Peter continually provided direction to virology and virologists that pushed both fields to explore common questions and new ideas. Peter always saw, discussed and explored those common themes that led us to answer those fundamental processes of what it took to reproduce oneself. At the end of the 1970s, we used those DNA tumor viruses as a tool to uncover a cellular protein, p53, which was an anti-oncogene or a tumor suppressor gene. It is clear that the tumor viruses played an important role in uncovering our present day understanding of human cancers.

In addition to his scientific contributions, Peter contributed to the development of the field of virology so that it had outlets to communicate, so that it had standards to aspire to, so that it moved toward original directions guided by each generation of leaders of the field. His service to the community of virologists assured productivity, a style of leadership that was inclusive and soft, uncovering new questions and directions that guided the field, and always holding to personal and professional standards everyone admired and aspired to fulfill. This issue of Current Topics in Microbiology and Immunology is a fitting tribute to Peter Vogt and his continuing and enormous contribution to our field. Thank you Peter.