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"Molecular Characterization of 'p53 Family Network' in Human Head and Neck Cancer and Anti-EGFR Therapy"

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“Molecular Characterization of ‘p53 Family Network’ in Human Head and Neck Cancer and Anti-EGFR Therapy”

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Sinto Sebastian, Luciano Grammatica and Angelo Paradiso. Telomeres, Telomerase and Oral Cancer (Review). Int J Oncol. 2005 Dec;27(6):1583-96.

Manuscript B

Sinto Sebastian, Jeffrey Settleman, Stephan J. Reshkin, Amalia Azzariti, Antonia Bellizzi, Angelo Paradiso. The complexity of targeting EGFR signalling in cancer:From expression to turnover. Biochimica et Biophysica Acta. Review on Cancer. 1766 (2006) 120–139.

Manuscript C

Sinto Sebastian, A. Azzariti, R. Accardi, D. Conti, B. Pilato, R. Lacalamita, L. Porcelli, G.M. Simone, S. Tommasi, M. Tommasino and A. Paradiso. Validation of gefitinib effectiveness in a broad panel of head and neck squamous carcinoma cells. Int J Mol Med. 21 (2008) 809-817.

Manuscript D

Wild type p53 role in cancer cell survival and proliferation, Manuscript under preparation.

ABSTRACT

Understanding of p53 family protein (p53, p63 and p73) networks in head and neck squamous cell carcinoma (HNSCC), can positively influence in cancer screening, diagnosis, treatment and prevention. P53 family proteins are regulating diverse cell signalling pathways at diverse conditions to determine cell fate. At each condition of cells state, how these proteins are controlling cell fate is more complexed due to the presense of several isoforms for each p53 family proteins and their multifaceted interactions. Like other solid tumours, the p53 pathway is disabled by several mechanisms in HNSCC. Despite of the convincing evidence of a high frequency of p53 mutations in HNSCC, a subset of cancers arise in the absence of mutations. The molecular mechaninsms through which p53 lacking mutations subvert its tumor supressor functions in HNSCC still remain uncertain. In fact, some cancers and established cancer cell lines are over-expressing wild type p53 protein makes questionable of p53 role as only a tumor suppressor or it has some other additional functions, even in cancer cells. For an answer of this hypothesis, we have performed detailed molecular characterization, in an invitro model of head and neck cancer, in a broad panel of 12 newly established HNSCC cell lines. In our studies, we found that some head and neck cancer cell lines are accumulating wild p53 protein hyperphosphorylated at serine15 and 392 and it leads to the over expression of ΔNp73, the mechanism already reported from our laboratory in HPV38 immortalized keratinocytes. To better understand the functions of accumulated wild p53 role in HPV positive and negative cancer cell lines, we have performed p53 knock down by siRNA and the results have showed that p53 knock down inhibited cell proliferation in both HPV positive and negative cancer cells. Moreover, p53 knock down induced significant morphological changes and senescence associated beta galactosidase activity in these cells. Our results pinpoint, wild-type p53 protein accumulated in transformed cell lines has an additional role in cell proliferation other than its well known tumor supressor activity. Moreover, the key role of p53 family network in modulating epidermal growth factor receptor (EGFR) expression and in controlling cell proliferation and apoptosis arises the question of whether p53 family proteins status influences the efficacy of EGFR inhibitors, investigating its ability to reduce cell growth, to induce apoptosis and to modulate cell cycle and various EGFR pathwayrelated targets in head and neck cancer. Because, recently improved understanding of the pathogenesis of human head and neck squamous cell carcinoma has led to the development of new, molecular based therapeutic strategies, one of the most promising is the utilization of tyrosine kinase (TK) inhibitors, targeting EGFR. The comparison between the targets analysed and gefitinib effectiveness evidenced the absence of a clear relationship, exluding them as predictive factors for gefitinib efficacy. Our results confirmed the in vitro efficacy of an anti-EGFR approach, but other targets than those analysed here should be characterized in order to identify valid predictive factors for gefitinib utilization in head and neck cancer. We

have also performed chemoresistance analysis in our invitro model of HNSCC cell lilnes and found that p53 family network has less predictive role in HNSCC chemoresistance but ABCG2, a multidrug resistance protein, has over-expressed in HNSCC.

1. BACKGROUND

1.1 Cancer

It is widely known that cancer is one of the most frequent causes of death, especially in the developed countries of the world, where the most common lethal diseases have now been practically eradicated. For cancer to reach a stage at which it causes serious damage and, potentially, death, it must follow a well-known pre-determined course. It emerges locally at cellular level, spreads and infects neighboring tissues, perhaps re-instantiates at a remote location using natural transport means and, if no action is taken to remove it or halt its growth, grows to a degree where the functionality of vital organs is irreversibly compromised. In the last few decades understanding of the molecular events at cell level, which are necessary for development of cancer, has greatly increased. According to a theory of carcinogenesis, a complex and multi-step process is likely involved in the development of cancer.

A number of factors are attributed to the development of cancer. The factors include diet, toxins, smoking, alcohol, obesity and infections, which usually help in the process of carcinogenesis by inducing variation in epigenetic code or cause aberrations in tumor suppressor genes, and oncogenes and genes involved in repair machinery. This usually leads to uncontrolled growth by recessive loss of tumor suppressor genes or dominant gain of oncogens and/or aneuploidy. Causative factors may vary among different cancers but the basic hypothesis that a normal human cell has to acquire at least six essential alterations to become a malignant cancerous cell in case of solid tumours. These six alterations include: the ability to sustain autonomous growth; the ability to avoid growth inhibitory signals; the evasion of apoptosis; the potential to replicate infinitely; sustained angiogenesis; and finally, for the malignant tissue to be invasive and metastatic (Hanahan and Weinberg 2000). Cancer cells contain many altered and/or mutated genes. These almost always include: mutations in genes that are involved in mitosis; that is, in genes that control the cell cycle. Their mutated or over-expressed products stimulate mitosis even though normal growth signals are absent. Example: *many tyrosine kinase receptors* including epidermal growth factor receptor (*EGFR)*, the gene encoding the receptor for epidermal growth factor (EGF) (*EGFR* is also known as *HER1*.) Moreover, for altering cell homeostasis in favor of cellular immortality cell has to by bass cell signals, which inhibit uncontrolled cell proliferation, they are tumor suppressor genes, and these genes normally inhibit mitosis. Example: the *p53* gene product normally senses DNA damage and either halts the cell cycle until it can be repaired or, if the damage is too massive; triggers apoptosis. During the course of cellular immortalization different cell signaling pathways are altered in favor of tumorogenesis and understanding of these altered cell signaling pathways in detail helps to design anticancer therapies.

1.2 p53

p53, (53KD) also known as tumour protein 53 (TP53), is a transcription factor that regulates the cell cycle and apoptosis, in case of cellular insults, and hence functions as a tumor suppressor. p53 has been described as "the guardian of the genome", "the guardian angel gene", or the "master watchman", referring to its role in conserving stability by preventing genome mutation. Briefly, p53 was first identified in 1979 as a cellular protein that bound to the simian virus (SV40) large T antigen and accumulated in the nuclei of cancer cells. The gene encoding p53 (TP53) was cloned from neoplastic rodent and human cells, and found to have weak oncogenic activity when expressed in rodent cells. In the late 1980s, however, researchers discovered that they were studying missense mutants of the TP53 gene instead of the wild-type gene. The missense mutations found in the original TP53 cDNA clones proved to be the key to understanding the pathobiological activity of p53. The ability of p53 to form tetramers allows this protein to behave in a dominant-negative fashion, where the allele-producing mutant p53 suppresses the activity of wild-type p53. Later its character as a tumor suppressor transcription factor was finally revealed in 1989 by Bert Vogelstein working at Johns Hopkins School of Medicine. Warren Maltzman, of the Waksman Institute of Rutgers University first demonstrated that TP53 was responsive to DNA damage in the form of ultraviolet radiation (Maltzman et al. 1984).

The transcription factor p53 responds to diverse cellular stresses to regulate target genes that induce cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. In addition, p53 appears to induce apoptosis through nontranscriptional cytoplasmic processes. In unstressed cells, p53 is kept inactive essentially through the actions of the ubiquitin ligase MDM2, which inhibits p53 transcriptional activity and ubiquitinates p53 to promote its degradation. Numerous posttranslational modifications modulate p53 activity, most notably phosphorylation and acetylation. Several less abundant p53 isoforms also modulate p53 activity. Activity of p53 is ubiquitously lost in human cancer either by mutation of the p53 gene itself or by loss of cell signaling upstream or downstream of p53

1.3 p63 and p73; members of p53 family

The identification of the two p53-related genes, p63 and p73, initially provoked speculation that all three genes might play an analogous role in human tumors (Yang and McKeon 2000; Melino et al. 2003; Moll and Slade 2004). In particular, the striking homology among the family members within both their DNA binding domain (DBD) and oligomerization domain (OD) suggested that these genes might regulate transcription of a common subset of target genes, by binding to common promoters as either homo- or heterotetrameric complexes.

Work from year to year, however, has revealed a much more complex picture of the contribution of p63 and p73 to human cancer. The p53-related genes p63 and p73 exhibit significant structural homology to p53; however, they do not function as classical tumor suppressors and are rarely mutated in human cancers. Both p63 and p73 exhibit tissue-specific roles in normal development and a complex contribution to tumorigenesis that is due to their expression as multiple protein isoforms. The predominant p63/p73 isoforms expressed both in normal development and in many tumors lack the conserved transactivation (TA) domain; these isoforms instead exhibit a truncated N-terminus (DN) and function at least in part as transcriptional repressors. p63 and p73 isoforms are regulated through both transcriptional and post-translational mechanisms, and they in turn regulate diverse cellular functions including proliferation, survival and differentiation. The net effect of p63/p73 expression in a given context depends on the ratio of TA/DN isoforms expressed, on physical interaction between p63 and p73 isoforms, and on functional interactions with p53 at the promoters of specific downstream target genes. These multifaceted interactions occur in diverse ways in tumor-specific contexts, demonstrating a functional 'p53 family network' in human tumorigenesis. Understanding the regulation and mechanistic contributions of p63 and p73 in human cancers may ultimately provide new therapeutic opportunities for a variety of these diseases.

1.3.1 Gene structure of the p53 family transcription factors

Although p63 and p73 are the most recently identified p53 family members, a p63/p73-like gene is considered to be the ancestral gene of the family (Yang et al. 2002). The proteins encoded by these two genes are more structurally similar to each other than to p53; however, all three family members possess several conserved protein domains (Figure 1a and 1b). These include an N-terminal transactivation (TA) domain, a central DNA binding domain (DBD), and an oligomerization domain (OD) (Yang and McKeon 2000). The highest degree of homology among the three members is observed within the DBD (>60% amino-acid identity between p53 and both p63 and p73, and 85% amino-acid identity between p63 and p73), including conservation of all essential DNA contact residues (De Laurenzi and Melino 2000). This structural similarities allows p53 family proteins to interact physically and functionally make relevant in human cancer research.

Figure 1

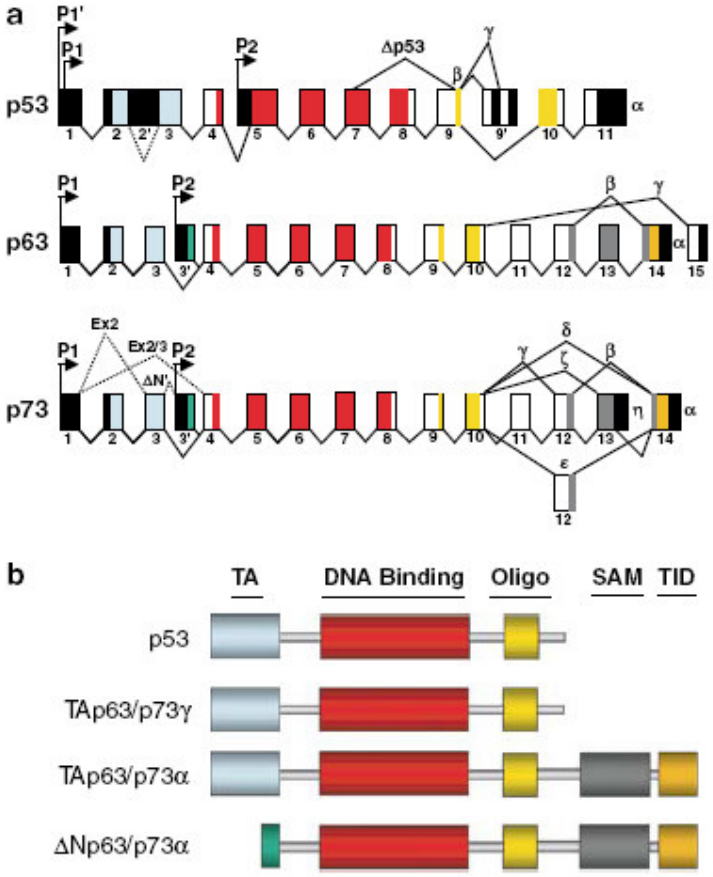


Figure 1. Structure and expression of p53 family members. (a) Structure of p53, p63 and p73 transcription units. Numbered boxes indicate exons, and black shading denotes untranslated sequences. The approximate regions encoding the transactivation (TA) domain (light blue), DN-specific region (green), DNA-binding domain (red), oligomerization domain (yellow), sterile alpha motif (SAM, grey), and transactivational inhibitory domain (TID, orange) are indicated. Distinct transcription start sites are indicated by arrows. N-terminal alternative splicing for p53 and p73 are indicated by dotted lines, and C-terminal splicing events for all p53 family members are indicated by solid lines and Greek letter designation.(b) Protein domains of p53 family members. All three family members share a homologous DNA-binding domain and oligomerization domain (oligo). The TA domain is shared by p53, TAp63, and TAp73 isoforms.TAp63 g/TAp73g isoforms most closely resemble p53.N-terminally truncated DN isoforms possess unique N-terminal sequences. Alpha isoforms of p63 and p73 possess a C-terminal SAM domain followed by a transactivational inhibitory domain (TID). Other isoforms of p53, p63 and p73 are not shown.

1.4 Role of p53; normal versus cancer cells

p53 is an intensively studied protein, its fame stemming mainly from its clear role as a tumour suppressor in humans and other mammals (Royds et al. 2006). Loss or mutation of p53 is strongly associated with an increased susceptibility to cancer, and most functions of p53 have been considered in the light of how p53 might protect from malignant progression (Vogelstein et al. 2000). Some *p53*-null mice can develop normally (Donehower 1996), an observation that has been taken to rule out major functions for p53 in normal physiology. But recent studies are questioning whether p53 is truly such a single-minded protein, and other functions of p53 that might be profoundly important during normal life are being uncovered. These include roles for p53 in regulating longevity and ageing, glycolytic pathways that might determine endurance and overall fitness, and apoptotic responses during ischaemic and other types of stress. Evidence for genetic variations in the activity of the p53 pathway in humans gives these ideas extra relevance (Pietsh et al. 2006). One of the major mechanisms by which p53 functions is as a transcription factor that both positively and negatively regulates the expression of a large and disparate group of responsive genes (Lapenko et al. 2006). Although some of these p53-responsive genes have an important role in mediating cell-cycle arrest, senescence and apoptosis (the best understood activities of p53), it is now evident that the ability of p53 to influence gene expression has wider reaching effects. Numerous studies, including the recently reported genome wide ChIP analyses (Cawley et al. 2004; Wei et al. 2006), have identified p53-regulated genes that could have a role in a number of different and sometimes unexpected responses. Although some of these still need to be fully validated, there is now clear evidence for a role of p53 in the regulation of glycolysis (Matoba et al. 2006; Bensaad et al. 2006) and autophagy (Crighton et al. 2006), the repair of genotoxic damage (Gatz and Wiesmuller 2006), cell survival and regulation of oxidative stress (Bensaad and Vousden 2005), invasion and motility (Roger et al. 2006), cellular senescence (Kortleverm et al. 2006), angiogenesis (Toeodoro et al. 2006), differentiation (Murray-Zmijewski et al. 2006) and bone remodeling (Wang et al. 2006). The cellular pathways in which p53 is involved, are schematically represented in Figure 2. In these aspects, its worthy to analyze there is any cancer cells are expressing wild type p53, and if they are expressing, its role in cancer cells has to be studied before clinical use of p53 mediated gene therapy as a canticancer therapy.

Figure 2

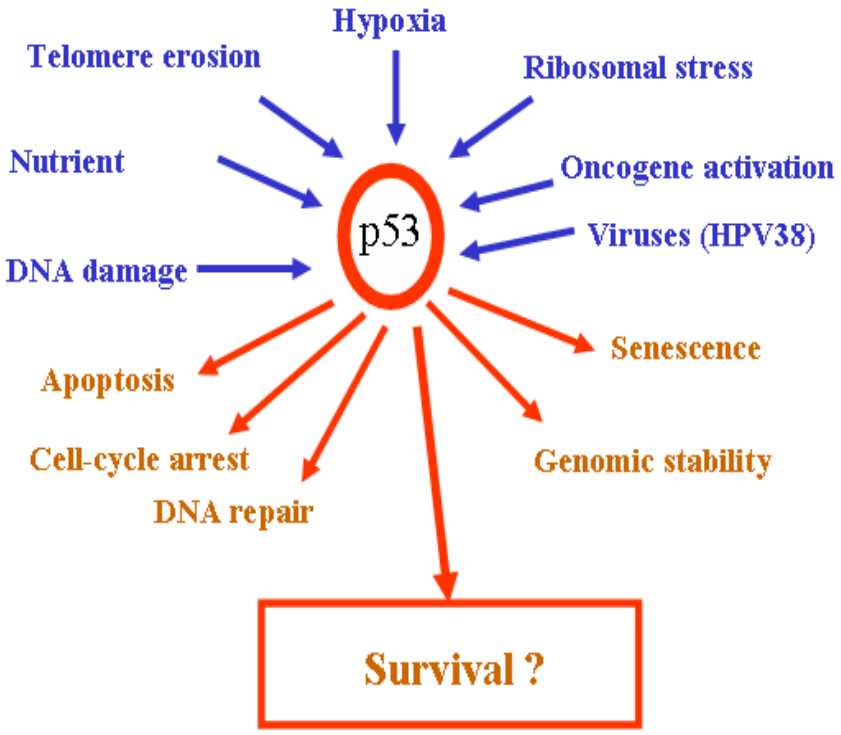


Figure 2. Activation and functions of p53. p53 has a key role in integrating the cellular responses (pink arrows) to different types of stress (blue arrows). Activation of p53 can result in a number of cellular responses, and it is possible that different responses are induced by different stress signals. There is evidence that p53 can play a part in determining which response is induced through differential activation of target-gene expression. Although the importance of these responses to tumour suppression is clear, previously unanticipated contributions of these responses to other aspects of human health and disease are being uncovered. The role of p53 in tumour suppression, development and ageing is likely to depend on which cellular response is activated and on the context in which the activation occurs.

1.4.1 A positive role; p53 functions in normal cells

Tumor suppressor function of human p53 protein has been well known but at what conditions and which state exerts its tumor suppressive property in genomic altered cells are unknown. It has become evident that despite the many levels of negative regulation that are in place to restrain p53, the everyday rigours of normal mammalian life can more systemically induce low levels of p53 activity. And, recent studies have revealed a hitherto unappreciated importance of p53 under conditions of apparently normal growth and development. Interestingly, induction of p53 through these mechanisms seems to have a role in responses beyond cell-cycle arrest and apoptosis, including an

intriguing role of p53 in promoting cell survival (Lassus et al. 1996) through regulation of glucose metabolism and antioxidant function as well as autophagy.

1.4.1.1 Regulation of glucose metabolism

Recent results have indicated a role for p53 in determining the response of cells to nutrient stress and in regulating pathways of glucose usage and energy metabolism. Metabolic stress that results in low glucose levels has been shown to activate p53 through a pathway that involves AMP kinase (AMPK), and has been proposed to contribute to the short-term survival of cells suffering, hopefully temporary, starvation (Jones et al. 2005). Such a starvation-induced activity of p53 is entirely consistent with the concept of p53 as a sentinel in the detection and response to potentially oncogenic stress, but could clearly have a much broader role in the response to metabolic stress. More surprisingly, roles for p53 in controlling different metabolic pathways under apparently normal growth conditions have also been recently described. For example, p53 has been shown to induce the expression of the copper transporter SCO2, which is required for the assembly of cytochrome *c* oxidase (Matoba et al. 2006). This allows p53 to enhance oxidative phosphorylation. Conversely, the loss of p53 activity in cells results in a reduction in oxygen consumption. The resultant defect in respiration would affect tumor cells (which are all defective in some way for p53 activity) particularly strongly, because the abnormal and deregulated proliferation that is characteristic of cancer cells makes them particularly energy demanding. In cancers, a dramatic increase in glycolysis called the 'Warburg effect' might help solve this problem of sustained energy production. Interestingly, loss of p53 seems to be a root cause of the metabolic changes that characterize cancer cells, because restoration of *SCO2* expression in p53- deficient cancer cell lines can restore mitochondrial respiration (Matoba et al. 2006). But what effect does this change in mitochondrial respiration have on the whole animal? Closer examination of *p53*-null mice led to the identification of an interesting, and hitherto undetected, defect in endurance. *p53*-null mice get exhausted very quickly during exercise presumably because they cannot efficiently generate energy through aerobic respiration (Matoba et al.2006) . This might be one of many cancer-independent phenotypes that result from the loss of p53 that have been overlooked owing to our extreme focus on tumor development.

1.4.1.2. Antioxidant functions

Another recently described p53-inducible gene with a role in glycolysis is *TIGAR* (Tp53-inducible glycolysis and apoptosis regulator) (Bensaad et al. 2006), which encodes a protein that shows some structural similarity to the bisphosphatase domain of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose 2,6 bisphosphate (PFK2/FBPase-2), and can lower the

intracellular levels of fructose-2,6-bisphosphate. An effect of TIGAR expression is therefore to decrease the activity of 6-phospho-1-kinase, a key glycolytic enzyme, thereby diverting the major glycolytic pathway into the pentose phosphate pathway. The resultant increase in nucleotide and NADPH production might have a number of interesting consequences, including an increase in glutathione levels to promote scavenging of reactive oxygen species (ROS). Although the apoptotic activity of p53 is mediated at least in part through increasing ROS levels (Johnson et al. 1996; Polyak et al. 1997), a number of studies have shown a survival function for p53 in lowering intracellular ROS levels, involving the activity of p53-inducible genes such as *TIGAR*, sestrins (Velasco-Miguel et al. 1999), aldehyde dehydrogenase-4 (*ALDH4*) (Yoon et al. 2004), and others (Gu et al. 2006). Most interestingly, this antioxidant function of p53 is important in the absence of acute stress and acts to prevent the accumulation of DNA damage during normal cell functions (Sablina et al. 2005). It is worth pointing out, however, that even among these low-stress p53-response genes, the sensitivity to p53 can differ. For example, SCO2 and sestrin levels are reduced by the removal of p53 even in unstressed cells, whereas basal *TIGAR* expression is not so clearly affected. It seems likely that rather than dividing into two discrete groups of genes that respond to high stress and low stress, p53-responsive genes will form a continuum that shows ever-increasing sensitivity to p53 and stress levels. Based on current evidence, we can propose a model in which p53 can have two important, but fundamentally opposing, roles in response to stress. The low levels of DNA damage that are encountered during normal life are dealt with, through p53, by lowering ROS levels (and so reducing damage) and by promoting the survival of the slightly damaged cell to allow repair (a process to which p53 can also contribute). In response to more severe, sustained stress such as oncogene activation or exposure to high doses of radiation p53 switches from promoting survival and repair to the induction of apoptosis (Bensaad et al. 2005). Of course, both activities of p53 (survival and apoptosis) contribute to tumor suppression. The question that has not been fully explored yet is: are there other consequences of loss of p53, in cancer cell which are expressing wild type p53 protein?

1.4.1.3 p53 in development

In vertebrates, the two other p53 family members, p63 and p73, illustrate a requirement for at least some p53-like function during development (Murray-Zmijewski et al. 2006). Deletion of either p63 or p73 has severe effects on the normal development of mice, and a number of human developmental diseases have also been linked to mutations in p63 (Barbieri et al. 2006). Although loss of p63 and p73 can result in a predisposition to cancer development (Flores et al. 2005), neither protein has the profound tumor-suppressive activity that is shown by p53. In light of the importance of p63 and p73 to normal development in mice, it is perhaps not so surprising that a closer analysis of the

p53-null mice revealed that they also show developmental abnormalities (Chio et al. 1999). Although many of these mice are normal at birth, females of some strains show neural tube-closure defects (or exencephaly) that reveal a role for p53 in normal development, at least under certain circumstances (Armstrong et al. 1995; Sah et al. 1995). This phenotype is also seen in mice with defects in other components of mitochondrial death pathways, which indicates that the lack of p53 leads to a failure in progenitor cell apoptosis and so an overproduction of neural tissue. Furthermore, studies of developmental abnormalities induced by *in utero* exposure to ionizing radiation clearly show that p53 has a role in reducing the rate of birth defects (Armstrong et al. 1995; Hall and Lane 1997; Baatout et al. 2002). These findings imply that the normal p53 response constantly monitors the early developmental process, presumably eliminating the odd aberrant cell or killing the embryo when the defects become too extreme (Hall and Lane 1997). Interestingly, p53 has a clear anti-teratogenic function (Nicol et al. 1995; Norimura et al. 1996), presenting a much more tangible evolutionary advantage than the more frequently studied anti-tumor activity and illustrating an interesting parallel with the germline functions of p53 in lower organisms. It would be interesting to know whether birth defects are more common in patients with *Li–Fraumeni syndrome*, who carry a germline mutation in one p53 allele and have an extremely high incidence of cancer (Varley 2003).

1.4.2 Negative role of p53 in human cells (The darker side of p53)

Although suppression of cancer or inhibitions of teratogenesis are clearly desirable features of p53, the induction of p53 is not without cost. Most clearly, p53 is strongly activated in response to acute genotoxic stress such as is encountered following irradiation or chemotherapy, and the ensuing apoptosis is responsible for the classic symptoms of radiation sickness and side effects of cancer therapy. It is also clear that other forms of stress or trauma, not necessarily associated with potential malignant progression, can also lead to the activation of p53. For example, induction of p53 during ischaemia has been shown to contribute to damage through the activation of apoptosis, and a temporary inhibition of p53 function under these conditions might be highly beneficial in the prevention and management of injury to the liver, brain and kidneys (Georgiev et al. 2006; Fiskum et al. 2004; Dagher 2004), or in treatment following myocardial infarction (Matsusaka et al. 2006). Equally tantalizing is the possibility that p53 plays a part in neurodegenerative syndromes such as *Parkinson's disease, Alzheimer's disease* and *Huntington's disease* (Jacobs et al. 2006). In contrast to the positive role of p53 in protection from cancer, each of these examples reflects a more negative role for p53 in which induction of the p53 response causes, rather than solves, problems. The concept that p53 has a darker side becomes even more profound with the realization that despite its help in protecting from cancer, p53 might also contribute to many of the undesirable aspects of ageing. Taken together, it

seems possible that we pay a high price for the protection from tumor development that is provided by p53.

1.4.2.1 p53 and ageing —the quest for eternal life

How p53 might promote ageing is not yet clear, although a contribution of p53 to cellular senescence and the limitation of the proliferative capacity of stem cells has been proposed (Sharpless and DePinho 2004). In Drosophila melanogaster, the extended lifespan that results from a reduction of p53 activity in neurons is not further enhanced by calorie restriction (Bauer et al. 2005), which increases longevity in a number of organisms from yeast to mice. These results indicate that p53 functions in the pathways that respond to caloric restriction such as those involving silent information regulator-2 (SIR2) and/or insulin signaling (Maier et al. 2004; Bauer and Helfand 2006). How these observations link to the function of p53 as a survival factor in response to glucose deprivation is not yet clear. Some of these results also hint at a possible role for the recently identified N- or C-terminally truncated isoforms of p53 (Bourdon et al. 2005) in controlling ageing (Tyner et al. 2002; Maier et al. 2004). Regulation of glycolysis by p53 can also affect cellular, and so potentially organismal, lifespan (Kondoh et al. 2005). However, despite the evidence that p53 can induce premature ageing, it seems that this might not be a function of properly regulated p53. Mice containing an extra copy of the p53 gene, or with reduced expression of Mdm2, showed the expected protection from tumor development but no decrease in normal lifespan (Garcia-Cao et al. 2002; Mendrysa et al. 2006). Although not fully resolved, one explanation for this apparent paradox is that the ageing phenotype observed in the earlier studies reflects an imbalance of p53 signalling, with the activation of some, but not all, p53 functions (Mendrysa and Perry 2006; Poyurovsky and Prives 2006). Although some activities of p53 would be predicted to contribute to accelerated ageing, it seems equally possible that lack of p53, and the associated enhanced oxidative stress, would also have the same effect. Indeed, a deficiency of the p53-related protein p63 has been shown to result in premature ageing that correlates with an induction of senescence (Efeyan et al. 2006). A recent study in the fruitfly has also indicated that p53 is required for compensatory growth after tissue damage and might contribute to tissue repair, cell renewal and survival in other animals (Wells et al. 2006). Indeed, mice expressing altered forms of p53 also show perturbed wound healing (Tyner et al. 2002).

1.5.The role of p53 in human cancer

In the two decades since its original discovery, p53 has found a singularly prominent place in our understanding of human cancer. Although the biochemistry of p53 has been worked out in some detail, our knowledge of the biologic consequences of p53 dysfunction is still quite rudimentary. P53

dysfunction in cancer cells are manly due to its mutation (50%), epigenetic modulation at expressional level and low persistence of p53 protein level due to its enhanced turnover. Most p53 mutations found in human cancers are not null mutations but rather encode mutant version of the p53 protein that may have unwanted activities such as a gain-of-function or be dominant negative inhibitors of wt p53 activity. In this regard, it will be important to determine how best to harness the complex properties of p53's ability to induce cellular growth arrest and cell death to generate novel, effective approaches to cancer therapy. Furthermore, a clearer appreciation of the direct interaction of epigenetic factors with p53 will lead to development of strategies to inhibit tumor initiation and progression.

1.5.1 p53; the guardian of the genome

DNA damage was the first type of stress found to activate p53 and, based on this, p53 has been widely regarded as "the guardian of the genome" (Lane 1992). Extensive characterization of the signaling routes that connect DNA damage with p53 have identified a cascade of Ser/Thr kinases that includes ATM, ATR, Chk1 and Chk2, which phosphorylate p53 (Siliciano et al. 1997; Chao et al. 2000; Saito et al. 2002; Kurz et al. 2004). This signaling cascade is permanently activated in human cancer, suggesting that the cancerous state is intrinsically associated to the generation of DNA damage (Bartkova et al. 2005; Gorgoulins et al. 2005). The constitutive DNA damage present in cancer cells is thought to emanate primarily from the strong generation of reactive oxygen species, (Jackson et al. 2001) as well as, from the aberrant firing of DNA replication origins (Bartkova et al. 2006; Di Micco et al. 2006). Recent characterization of mice genetically manipulated with a knocked-in p53 that cannot be phosphorylated at two of the main residues targeted by ATM/ATR/Chk1/Chk2, namely, Ser18 and Ser23 (Ser15 and Ser20 in human p53), indicates an important role of these phosphorylation sites in some, but not all, the DNA damage induced and p53-dependent responses (Chao et al. 2006). In agreement with this, mice carrying p53S18A/S23A alleles are tumor prone, (Chao et al. 2006) although this phenotype is considerably milder than in the case of p53-null mice (Donehower et al. 1992; Jacks et al. 1994). These data suggest that the activation of p53 in response to DNA damage occurs through multiple pathways, which in addition to the well-established kinase cascade of ATM/ATR/Chk1/Chk2, probably include other kinases such as p38, JNK/SAPK and c-Abl (Milne et al. 1995; Hu et al. 1997; Fuchs et al. 1998; Bulavin et al. 1999; Buschmann et al. 2001; Harris et al. 2005). Regarding human cancer, the available information gathered from the analysis of epi-genetic aberrations indicates that the aforementioned DNA damage signaling kinases are not, in general, significant targets of genetic and epi-genetic inactivation (Ingvarsson et al. 2002; Bartek et al. 2003; Feng et al. 2003). The only exception to this is found in hematological malignancies, which present a high incidence of mutations in ATM (13–40% depending on the particular type

of malignancy) (Gumy-Pause et al. 2004). In line with this, a recent large-scale sequencing effort of 210 diverse human cancers has identified ATM among the three most frequently mutated kinases (5% incidence) (Greenman et al. 2007). Based on the above genetic evidence, it can be concluded that DNA damage is conveyed to p53 through multiple redundant pathways in which many transducers participate, but none of them plays a critical role and, therefore, alteration of a single component does not have a significant impact on p53 function.

1.5.1.1. p53; The policeman of the oncogenes

Among the many and varied stimuli that have been reported to activate p53, oncogenic signaling (Serrano et al. 1997) has gained much attention because, as DNA damage, is also universally present in cancer. Therefore, analogous to the title of "guardian of the genome", we can also assign to p53 the function of "policeman of the oncogenes". Oncogenic signaling activates p53 through ARF, (Palmero et al. 1998; de Stanchina et al. 1998; Zindy et al. 1998) which, in turn, interacts with MDM2 inhibiting its p53-ubiquitin ligase activity. In this manner, ARF-dependent stabilization of p53 results in a dramatic increase in p53 activity. Many transcription factors activate ARF in response to oncogenic signaling, (Sharpless 2005; Gil 2006) most notably, Dentin matrix acidic phosphoprotein (DMP1) (Inoue et al. 1999; Inoue et al. 2000). Mice lacking ARF have a remarkable tumor-prone phenotype, (Kamijo et al. 1997; Kamijo et al. 1999) although not as severe as p53-deficient mice, (Donehower et al. 1992; Jacks et al. 1994) and there is good genetic evidence in mice supporting the relevance of the ARF/MDM2/p53 axis in tumor suppression (Moore et al. 2003). Importantly, mice deficient in ARF present a normal DNA damage response, indistinguishable from ARF-proficient mice (Kamijo et al. 1999). Regarding human cancer, the analysis of (epi)genetic alterations indicate that ARF is indeed inactivated with an extraordinary high frequency (~30%) (Sharpless et al. 2005). However, inactivation of ARF almost invariably occurs in combination with the loss of p16INK4a, the cell cycle inhibitor, thus generating an ambiguity about which is the key targeted tumor suppressor. In this regard, it should be mentioned the existence of germline point mutations that inactivate ARF alone (i.e., sparing p16INK4a) in human kindreds predisposed to cancer (Randerson-Moor et al. 2001; Rizos et al. 2001). Nonetheless, the number of germline mutations that inactivate p16INK4a only (i.e., sparing ARF) outnumbers by a factor of ~20 those that inactivate ARF alone (Kim et al. 2006). Together, currently available evidence indicates that ARF is an important upstream regulator of p53, whose lack of activity has a significant impact on cancer.

1.5.2 Downstream events of the p53 pathway

Once the p53 protein is activated, it initiates a transcriptional program that reflects the nature of the stress signal, the protein modifications and proteins associated with the p53 protein. The p53 protein binds to a specific DNA sequence, termed the p53- responsive element (RE) (EI-Deiry et al. 1992; Bourdon et al. 1997), and induces the expression of downstream genes. An algorithm that identifies p53-responsive genes in the human and mouse genome has been utilized to detect a number of new genes regulated by the p53 protein (Hoh et al. 2002). The genes in this p53 network mainly initiate one of three programs that result in cell cycle arrest, DNA repair or apoptosis.

1.5.2.1 Growth arrest

p21*WAF1*/*CIP1* is known to be a p53-downstream gene, and has been suggested to mediate p53-induced growth arrest triggered by DNA damage. The p21 protein is a cycline-dependent kinase inhibitor that associates with a class of CDKs and inhibits their kinase activities. This will facilitate the accumulation of hypophosphorylated form of pRB that in turn associates with E2F inhibiting its transcriptional activity, leading to cell cycle arrest. As long as pRb is bound to E2F, the cell is prevented from entering into S phase. This G1 arrest affords the cell time to repair the DNA damage. Should repair be unsuccessful, P53 levels drop and CDK-cyclin protein kinase activity resumes, leading to entry into S phase. In the event that the DNA is not repair, p53 triggers apoptosis (Makoto et al. 1999; Kim et al. 2005).

1.5.2.2 DNA repair

Soon after having established TP53 as the most frequently altered gene in human tumours in the 1990s, (Hollstein et al. 1991, deFromentel and Soussi 1992) p53 was understood as a major component of the DNA damage response pathway (Lane 1992; Levine 1997). After the introduction of DNA injuries the level of p53 protein rises, which in turn induces a transient cell cycle arrest or apoptotic cell death. DNA damage activates p53 through post-translational modifications by specific kinases, such as the strand break sensor ataxia telangiectasia mutated protein (Atm), by acetyltransferases like CREB-binding protein (Cbp)/p300, and by the poly (ADPribose) polymerase 1 (Parp-1), which prevent proteolysis via the Arf-mouse double minute 2 (Mdm2) pathway and/or enhance binding of p53 to consensus sequences within the genome (Saito et al. 2003; Vaziri et al. 1997; Wang et al. 1998). Initially, investigations on a direct participation of p53 in DNA repair were spurred by a number of biochemical observations. Thus, the C-terminal 30 amino acids of p53 were shown to recognize several DNA damage-related structures, such as DNA ends, gaps, and insertion/deletion mismatches (Bakalkin 1994; Jayaraman and Prives 1995; Lee et al. 1995; Reed et al. 1995). p53 was also demonstrated to catalyse reannealing of short stretches of single- and double-stranded DNA and to

promote strand exchange between them (Bakalkin et al. 1994, Oberosler et al. 1993, Jean et al. 1997). Further, p53 binds to three-stranded heteroduplex joints and four-stranded Holliday junction DNA structures with localization specifically at the junction, suggesting that p53 directly participates in recombinational repair (Lee et al. 1997; Dudenho¨ ffer et al. 1998; Janz et al. 2002). Moreover, several groups demonstrated a Mg2þ-dependent 3'–5' exonuclease activity intrinsic to p53 (Jean et al. 1997; Bakhanashvili 2001). Noticeably, the same central region within p53, where tumourigenic mutations are clustered, recognises DNA sequence specifically, is required for junction-specific binding of heteroduplex joints and is necessary and sufficient for the 3'–5' exonuclease activity on DNA (Mummenbrauer et al. 1996; Janus et al. 1999; Dudenho¨ ffer et al. 1999). In addition to p53's biochemical activities, numerous reports on physical and functional protein interactions further strengthened the proposal of a direct role of p53 in nucleotide excision repair (NER), base excision repair (BER), and double-strand break (DSB) repair (Albrechtsen et al. 1999; Bertrand et al. 2004; Sengupta and Harris 2005).

1.5.2.3 Apoptosis

Pivotal to the tumor-suppressor activity of p53 is its ability to activate apoptosis via multiple different pathways (Fridman et al. 2003). Since the most-studied function of p53 is its role as a transcription factor that can activate transcription of an ever-increasing number of target genes, its transcriptional activation of pro-apoptotic genes, as well as its transcriptional repression of anti-apoptotic genes, has been widely analyzed (Fridman et al. 2003 and Vousden et al. 2002). However, although a large number of genes regulated by p53 during induction of apoptosis are known (Fridman et al. 2003 and Vousden et al. 2002), no single target gene has been identified whose altered expression alone can sufficiently explain p53 mediated transcription dependent apoptosis, and whose genetic deficiency phenocopies p53 deficiency in vivo. As an additional mode of p53's pro-apoptotic activity, recent studies have placed non-transcriptional pro- apoptotic activities of p53 at the center of an active debate that aims to establish a comprehensive understanding of p53- mediated apoptosis.

1.5.2.3.1 p53 role in transcription dependent apoptosis

The past twenty-five years have seen intensive and varied investigations to better understand the functions that p53 uses to mediate apoptosis. The first indication of the role of p53 in apoptosis was obtained using the M1 mouse myeloid leukemia cell line lacking endogenous p53. Using M1 cells stably transfected with a temperature-sensitive mutant that acquires the conformation of wild-type p53 at permissive temperature (32˚C), it was observed that upon downshift to the permissive temperature, the transfectants underwent rapid loss of viability with the characteristics of apoptosis (Yonish-Rouach et al. 1991

and Selvakumaran et al. 1994). Several mechanisms have been implicated in p53-mediated apoptosis. One is p53 activation to up-regulation of pro-apoptotic Bax and down-regulation of pro-survival Bcl-2 (Selvakumaran M et al. 1994, Miyashita T et al. 1994). More recently its determined that p53-mediated apoptosis of M1 cells involves rapid activation of the pro-apoptotic Fas/CD95 death pathway-via up-regulation of membrane bound Fas (Vesely et al. 2006) and the intrinsic mitochondrial pathway, which results in activation of caspases 8, 9 and 10. Either Fas blocking antibody or inhibition of the apical caspases 8 and 10, were each almost as effective as IL-6 in abrogating p53 mediated apoptosis. These observations argue that p53 regulation of the bcl-2 members Bax and BcI-2, associated with the intrinsic mitochondrial apoptotic pathway, is ancillary to the extrinsic Fas/CD95 apoptotic pathway in mediating p53 induced apoptosis of M1 myeloid leukemia cells (Bennett et al. 1998). In other cell types up-regulation IGF-BP3, (Buckbinder et al. 1995) which sequesters the cell survival factor insulin-like growth factor-1 has been associated with p53 mediated apoptosis (Baserga et al. 1994). The gene encoding for the cathepsin-D protease, (Wu et al. 1998) PAG608 which encodes a nuclear zinc finger protein (Israeli et al. 1997) and the human homolog of the Drosophila sina gene (Nemani et al. 1996) have also been implicated as mediators of p53 induced apoptosis in various cell types. Furthermore, a series of p53-induced genes (PIG genes) were documented to encode proteins that respond to oxidative stress, suggesting that p53-mediated apoptosis involves activation of redox- controlling targets followed by increase in ROS, oxidative damage to mitochondria and caspase activation (Polyak et al. 1997). Along this research line it was recently observed that p53 suppresses Nrf2-dependent transcription of antioxidant response genes, presumably to prevent the generation of antioxidants that could hinder induction of apoptosis (Faraonio et al. 2006) Clearly established is p53's role as a nuclear transcription factor with the ability to activate, or repress, the expression of many genes. A number of p53 transcriptional targets, such as the p53-induced genes BAX, PUMA, NOXA, and the p53-repressed genes BCL2 and SURVIVIN, represent genes with the potential to promote or inhibit apoptosis, respectively, in stressed cells. Puma and Noxa are thought to indirectly induce mitochondrial outer membrane permeabilization (MOMP), known to be induced by the activation of Bax and Bak, via interfering with Bax and Bac interaction with prosurvival Bcl-2 family members (Moll et al. 2006). Interestingly, it was observed that Puma and Noxa differentially contribute to the regulation of p53-mediated apoptotic pathways. In normal cells, Puma was found to induce mitochondrial outer membrane permeabilization via an ER-dependent pathway; however, upon E1A oncoprotein expression, cells also became susceptible to mitochondrial outer membrane permeabilization induction by Noxa via an ER-independent pathway (Tsukasa Shibue1 et al. 2006). In several instances, transcriptional activation by p53 was observed to be dispensable for p53-dependent apoptosis, since mutants p53 which fail to activate transcription could still induce apoptosis (Chen et al. 1996 and Haupt et al. 1995). In addition, p53-dependent apoptosis could occur

in the presence of inhibitors of transcription and translation (Caelles et al. 1994). In recent years it has become clear that p53 also harbors a direct pro-apoptotic function at the mitochondria via engaging in protein-protein interactions with anti- and pro-apoptotic Bcl2 family members, including BclXL and Bak (Moll et al. 2006).

1.5.2.3.2 p53 role in mitochondrial mediated apoptosis

It has been reported, certain transcriptionally inactive mutants of p53 can still induce apoptosis when over expressed in tumor cells (Haupt et al. 1995; Chen et al. 1996). Also, in response to some stresses, such as hypoxia, p53 induces apoptosis but does not function as a transactivator (Koumenis et al. 2001). Intriguingly, Moll and colleagues demonstrated that during p53-dependent apoptosis a fraction cellular p53 protein localizes to mitochondria and induces cytochrome c release; however, this is not observed during p53-mediated cell cycle arrest (Marchenko et al. 2000). Additional support for the concept that p53 has a cytoplasmic role in apoptosis induction resulted from functional analysis of polymorphic variants of p53 (Dumont et al. 2003). Within exon 4 of the p53 gene, a common single-nucleotide polymorphism (SNP) at codon 72 leads to the incorporation of either an arginine (R72) or a proline (P72) at this position of the protein. Further investigation revealed that the R72 form of p53 induces apoptosis markedly better than the P72 variant (Dumont et al. 2003). When explored the potential mechanisms underlying the observed functional difference between the two p53 variants, made the initially surprising discovery that the greater apoptotic potential of the R72 form correlated with its much better ability to traffic to mitochondria. Based on these data, therefore concluded that the enhanced apoptosis-inducing activity of the R72 protein related, at least in part, to its greater mitochondrial localization. An analysis of whole cell or mitochondrial extracts by immunoprecipitation-western blot analysis, demonstrated the R72 form of p53 binds better to the mitochondrial death-effector protein BAK than does the P72 variant, correlating with the difference in apoptotic potential of the two p53 variants. In healthy cells, Bak resides at mitochondria as an inactive monomer. In response to various death stimuli, it undergoes an activating allosteric conformational change that promotes homo-oligomerization. This leads to formation of a pore in the outer mitochondrial membrane, and allows the release of cytochrome c and other pro-apoptogenic factors from the mitochondria resulting in the activation of a caspase cascade (Fig 3) (Griffiths et al. 1999; Wei et al. 2000). Recently, like BAK, the BCL2 family members BAX and BCL-XL have also been implicated in mitochondrial apoptosis induction by p53(Fig 3) (Mihara et al. 2003; Chipuk et al. 2004).

Figure 3

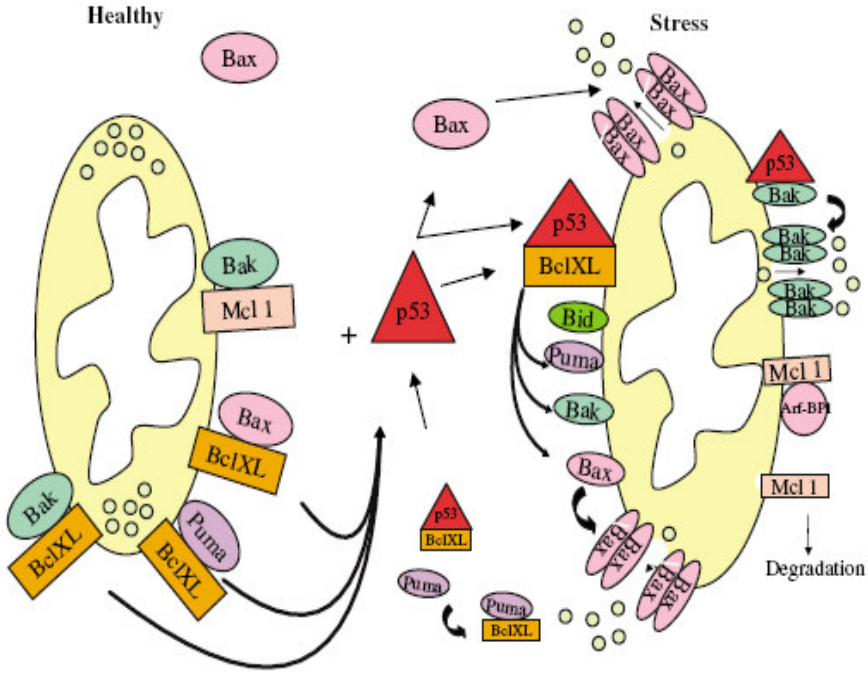


Figure 3. The mitochondrial pro-apoptotic activities of p53. In healthy cells (left), the pro-apoptotic BH3-only and effector BH123 proteins (ovals) exist either in complex with anti-apoptotic proteins Bcl2 family proteins such as BclXL, Bcl2 and Mcl-1 (rectangles). Death stimulus induced mitochondrial p53 (triangle) forms a complex with BclXL and Bcl2, liberating pro-apoptotic BH3-only proteins (derepression by inhibiting their survival function) and BH123 proteins, resulting in Bax and Bak homo-oligomerization, activation and mitochondrial membrane permeabilization (MMP) to release a host of apoptotic activators. Moreover, translocated p53 can bind to Bak by disrupting the inhibitory Bak/Mcl1 complex, enabling Bak to ultimately undergo homo-oligomerization and MMP. In addition, in cells with a cytosolic BclXL fraction, p53 might activate cytoplasmic Bax through a 'hit and run' mechanism involving an intermediate cytosolic p53/BclXL complex that is disrupted by cytosolic Puma, which results in Bax conformational change, mitochondrial translocation, oligomerization and MMP.

1.6 Modes of p53 alteration during tumorigenesis

Alterations in the gene encoding the cellular p53 protein are perhaps the most frequent type of genetic lesions in human cancer. At the heart of these alterations is the abrogation of the tumor suppressor activity of the normal p53. In many cases this is achieved through polymorphisms, and point mutations in p53, which often result in pronounced conformational changes. Such mutant polypeptides, which tend to accumulate to high levels in cancer cells, are believed to exert a dominant negative effect over co-expressed normal p53. Other than polymorphisms and mutation mediated inactivation, cellular signaling pathways are involved to inactivate expressed wild type p53 through

enhanced MDM2 mediated degradation pathway, over-expression of dominant negative regulators of p53 family isoforms, e.g. detlaNp73 and other post-translational modifications.

1.6.1 Polymorphisms in Tp53: the codon 72 polymorphism

The cDNA for p53 was first cloned in 1984; (Matlashewski et al. 1984; Wolf et al. 1985; Harlow et al. 1985) approximately 2 years later, a mobility shift in p53 protein was identified as a sequence polymorphism at amino acid 72, changing proline to arginine (Matlashewski et al. 1987; Buchman et al. 1988; Ara et al. 1990). Unfortunately, at the time of its discovery, researchers were inadvertently studying a tumor-derived mutant form of p53, with the belief that TRP53 was an oncogene. Therefore, the only functional analysis performed for these polymorphic variants was for their transforming potential, which did not differ, so the polymorphism was deemed functionally insignificant. Only in 1994, when analyzing the allele frequencies of the proline 72 and arginine 72 variants (P72 and R72, respectively) in human populations did Beckman and co-workers note statistically significant differences in allele frequency between different ethnic groups. For example, the P72 allele frequency is approximately 60% in African Americans, but only 30–35% in Caucasian Americans. Interestingly, Beckman noted that the P72 allele frequency increases in a linear manner in multiple populations as they near the equator (Beckman et al. 1994). This led him to hypothesize that the codon 72 polymorphism might have an impact on p53 function, and that the high exposure to UV light in populations near the equator led to selection for the P72 allele. The first comparison of the biological activity of endogenous P72 and R72 proteins was performed by Bonafe et al. in 2002; in that study, blood leukocytes homozygous for R72 were found to undergo significantly increased apoptosis in response to the cytotoxic drug cytosine arabinoside, compared to P72 (Bonafe et al. 2002). Further analysis, however, revealed that the R72 variant demonstrated greatly increased trafficking to mitochondria, where Moll and co-workers had reported in 2000 that p53 had direct ability to induce cytochrome c release and programmed cell death (Marchenko et al. 2000). A number of studies have attempted to determine if there is an association between codon 72 polymorphic variants of TP53 and risk for particular cancer types. These studies have come to very varied conclusions, with some studies reporting increased risk associated with the P72 allele for certain cancer types (Sjalander et al. 1996; Wu et al. 2002; Granja et al. 2004) and others failing to reach such conclusions (Weston et al. 1994; Birgander et al. 1995; Rosenthal et al. 1998). The reasons for these discrepancies are not clear, but it can be safely said that if such associations exist, they may not be particularly strong, or they can be influenced by unknown variables that presently are not controlled for in such studies.

1.6.1.1 The codon 47 polymorphism

A second exonic polymorphism at codon 47 changing a proline to serine has also been described (Felley- Bosco et al. 1993). The frequency is very low, ranging from 0.5% to 5% in various studies. This polymorphism is close to serine 46, phosphorylation of which is a key event for the apoptotic function of p53. In vitro studies have shown that the S47 variant is a poorer substrate for S46 phosphorylation and has an impaired proapoptotic ability (Li et al. 2005). The clinical significance of this p53 variant is not known.

1.6.2 p53 mutations in cancer

The p53 tumor suppressor gene is mutated in about half (50%) of all cancer types arising in a variety of tissues, and it manifests a high frequency of missense mutations (substitution of an amino acid in the encoded protein) (Hollstein et al. 1991; Levine et al. 1991; Aguilar et al. 1994). Non-missense mutations, e.g., deletions and insertions, are also found in the p53 gene but at a lower frequency—when compared with to other tumor suppressor genes, such as APC, BRCA and ATM. In the distribution of missense mutations, these types of mutations occur more frequently in exons 2–4 (54%) and 9–11 (77%) rather than in exons 5–8 (20%). The N-terminus of the p53 protein _encoded by exons 2–4 (Liu et al. 1993; Vogelstein et al. 1992; Thut et al. 1995; Lu et al. 1995), has an abundance of acidic amino acids that are involved in the transcriptional function of p53 (Raycroft et al. 1990; Fields et al. 1990), and binds to transcription factors such as TBP in TFIID (Liu et al. 1993; Seto et al. 1992; Truant et al. 1993; Martin et al. 1993; Mack et al. 1993). Experimental studies have shown that multiple point mutations in this domain are required to inactivate its transcriptional transactivation function (Lin et al. 1994). The carboxy-terminus _encoded by exons 9–11 of the p53 protein is enriched in basic amino acids that are important in: the oligomerization and nuclear localization of the p53 protein (Clore et al. 1994; Jeffrey et al. 1995; Hupp et al. 1995), the recognition of DNA damage (Jayaraman et al. 1995; Bakalkin et al. 1994), the negative regulation of p53 binding to promoter sequences of genes regulated by p53, the transcription of p53-transactivated genes (Horikoshi et al. 1995), and the induction of apoptosis (Wang et al. 1996). Laboratory studies have shown that at least 2-point mutations in the N-terminus of p53 are required to inhibit its transcriptional transactivity (Lin et al. 1994); therefore, deletions and insertions are more detrimental mutagenic mechanisms than single-point mutations for disrupting these N-terminal and C-terminal functional domains. More intensive studies on p53 mutation analysis find that some carcinogens are able to cause specific mutations.

1.6.2.1 Aflatoxin B exposure and p53 codon 249 ser 1 (AGG^{a}AGT)mutation

In liver tumors from persons living in geographic areas where aflatoxin B and hepatitis B virus (HBV 1) are cancer risk factors, the majority of *p53* mutations are at the third nucleotide pair of codon 249 (Bressac et al. 1991; Scorsone et

al. 1992; Li et al. 1993). A dose-dependent relationship between dietary aflatoxin B1 intake and codon 249ser *p53* mutations is observed in hepatocellular carcinoma from Asia, Africa and North America _reviewed in Refs.(Harris et al. 1996 and Montesano et al. 1997). In addition, the mutation load of 249ser mutant cells in non tumorous liver is positively correlated with dietary aflatoxin B exposure (Hussain et al. 1998). Exposure of aflatoxin B to human liver cells in vitro 1 produces 249ser_AGG to AGT) p53 mutants (Aguilar et al. 1993 and Mace et al. 1997). These results indicate that the expression of the 249ser mutant p53 protein provides a specific growth and/or survival advantage to liver cells (Puisieux et al. 1995), and are consistent with the hypothesis that *p53* mutations can occur early in liver carcinogenesis.

1.6.2.2 Sunlight exposure and p53 CC^{a}TT tandem double mutation

Sunlight exposure is a well known risk factor for skin cancer. Tandem CC to TT transition mutations are frequently found in squamous and basal cell skin carcinoma (Brash et al. 1991) while they are rarely reported in other types of cancers (Greenblatt et al. 1994). In vitro studies have shown the induction of the characteristic CC to TT mutations by ultraviolet exposure (Bredberg et al. 1986; Brash et al. 1988; Kress et al. 1992). Sunlight- exposed normal and precancerous skin contain CC to TT tandem mutations (Nakazawa et al. 1994 and Ziegler et al. 1994). These results indicate that CC to TT mutations induced by sunlight exposure may play a role in the occurrence of skin cancer.

1.6.2.3 Benzo[a]pyrene exposure and p53 mutations at hotspot codons 157, 248 and 273

Cigarette smoking has been established as a major risk factor for the incidence of lung cancer. Codons 157, 248 and 273 of the *p53* gene have been designated as mutational hotspots in lung cancer. The majority of mutations found at these codons are G to T transversions. Furthermore, in addition to lung cancer, codon 157 also constitutes one of the hotspots for G to T transversions in breast, and head and neck cancers. In smoking-associated lung cancer, the occurrence of G to T transversions has been linked to the presence of benzo(*a*)pyrene (BP) in cigarette smoke. Interestingly, codon 157 (GTC to TTC) mutations are not found in lung cancer from never smokers (Greenblatt et al. 1994; Hainaut et al. 1998). A dose-dependent increase in *p53* G to T transversion mutations with cigarette smoking has been reported in lung cancer (Takeshima et al. 1993). Recently, it is shown that BP diol epoxide, the metabolically activated form of BP, binds to guanosine residues in codons 157, 248 and 273 which are mutational hotspots in lung cancer (Denissenko et al. 1996). Cigarette smoke condensate or BP also neoplastically transforms human bronchial epithelial cells in vitro (Klein-Szanto et al. 1992). The occurrence of characteristic p53 mutation spectra upon exposure to a particular carcinogen can add to the 'weight of the evidence' for implicating an environmental pollutant-contaminant in the etiology of human cancer (Aguilar et al. 1994).

1.6.3 Co-operation and competition occur among p53 family isoforms

A complex picture emerges when we try to define the precise contribution of either p63 and/or p73 of 'p53 family network' to tumor development. A major part of this complexity stems from the expression of both DN and TA isoforms of p63 and p73 in many human tumors. Studies support the view that TAp63/TAp73 isoforms, like p53, exhibit tumor suppressive properties, and that up regulation of ΔNp63/ ΔNp73 isoforms is a common mechanism of their inactivation during tumorigenesis. Indeed, important physical and functional interactions among family members have now been demonstrated in tumor-specific contexts. These diverse interactions are mediated through two general mechanisms. First, cooperation and competition at the conserved p53 family binding sites within promoters of particular shared target genes are likely to regulate interactions among all three family members (Flores et al. 2002; Stiewe et al. 2002; Yang et al. 2006). Second, direct physical interaction of isoforms is known to alter the function of the tetrameric complex required for DNA-binding and transcriptional regulation (Chan et al. 2004). In ectopic expression studies, heteromeric complexes have been demonstrated between different isoforms of the same gene, between TAp63 and ΔNp73 isoforms, and between TAp73 and ΔNp63 isoforms (Chan et al. 2004). In each case, the respective DN isoforms function as potent inhibitors of transactivation by the respective TA isoforms. Consistent with these findings, endogenous complexes have also been demonstrated between different p73 isoforms, and between ΔNp63 and TAp73 (Deyoung et al. 2006; Rocco et al. 2006). Wild-type p53 binds p63 and p73 with much lower affinity than p63 and p73 bind one another (Davison et al. 1999). Despite the absence of a strong physical interaction, however, it seems highly plausible that in some tumor contexts both ΔNp63 and ΔNp73 serve to inhibit the function of p53 through promoter competition or other indirect mechanisms (Stiewe et al. 2002).

1.7 p53 post translational modifications

The well-studied post-translational modification of a single protein to date is human p53 protein. Therefore, several reports state its post-translational modifications role in determining its tumor-suppressor activity. And any alterations in it post-translational modifications can positively or negatively regulate cell fate determination and tumorigenesis.

1.7.1 Phosphorylation of serines and threonines

Human p53 has 23 different phosphorylation and dephosphorylation sites (Figure 4a). The regulation of p53 function by phosphorylation and dephosphorylation could, therefore, occur through many sites, most of which are outside the DNA binding domain (DBD). Most residues are phosphorylated by many different kinases in response to many stresses and are associated with

p53 activation (Bode et al. 2004). This defines two levels of potential redundancy, as a specific residue can be phosphorylated by several kinases (for example, serine 15 is phosphorylated by at least 8 kinases), and a specific kinase can phosphorylate several residues (for example, CHK2 phosphorylates 7 different residues). Such redundancy might provide a fail-safe mechanism to enable diverse stresses to activate p53 (Bode et al. 2004). As some residues seem to be phosphorylated by a single kinase, unique phosphorylation patterns might determine a subset of cellular responses. Alternatively, this could reflect incomplete knowledge of the relevant kinases and their targets. For example, serine 378 was thought to be phosphorylated by a single kinase just 2 years ago (Bode et al. 2004), but recent data indicate that three different kinases are involved (Ou et al. 2005). Additionally, the dephosphorylation of some residues has been correlated with activation; therefore serine 376 is phosphorylated in unstressed cells and dephosphorylated after ionizing irradiation, correlating with the interaction of p53 with 14-3-3 proteins (Stavridi et al. 2001). Conversely, the phosphorylation of serine 215 by Aurora kinase A reportedly inhibits the binding of p53 to DNA and overrides stress responses induced by cisplatin and χ-irradiation (Liu *et al*. 2004).

Figure 4

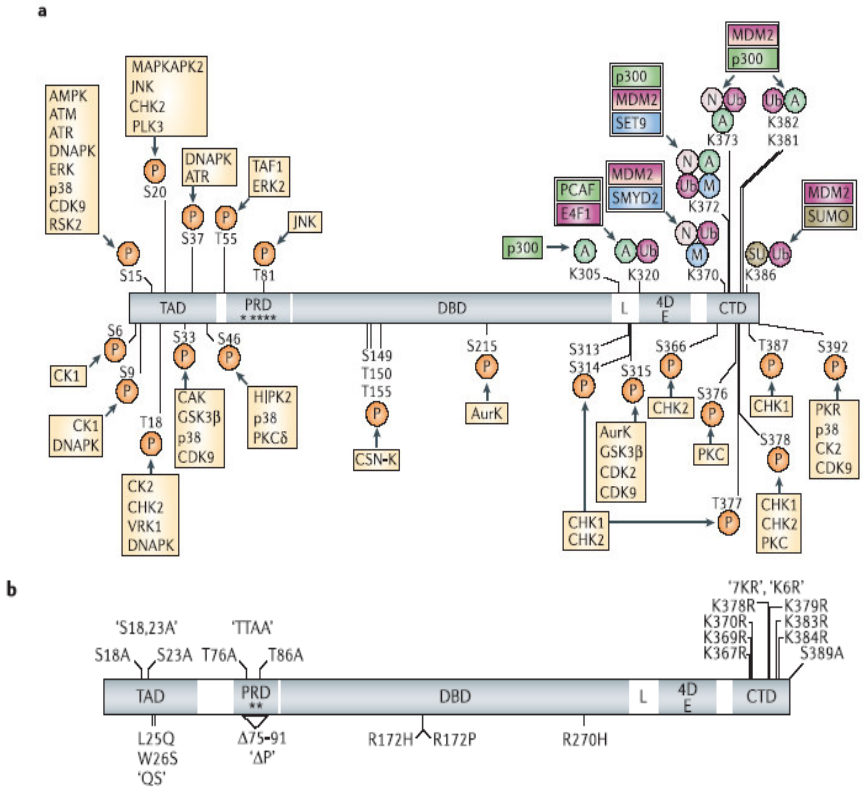


Figure 4. Comparative maps from *in vitro* human p53 and *in vivo* mouse p53 studies. a | Post-translational modifications of human p53. Specific residues are modified as shown, with phosphorylation (P) in orange, acetylation (A) in green, ubiquitylation (Ub) in purple, neddylation (N) in pink, methylation (M) in blue and sumoylation (SU) in brown. Proteins responsible for these modifications are shown in matching colours. b | Targeted mutations at the mouse p53 locus. Mouse p53 shares a strong homology with human p53, but a few differences can be noted, including: mouse p53 is comprised of 390 amino acids; the N-terminal part of mouse p53 is longer by 3 residues, so that the numbering is higher in the murine transactivation domain (TAD) than in the human TAD; the p53 proline-rich domain (PRD) is loosely conserved in evolution (the murine PRD is shorter, and contains 2 PXXPs motifs and 2 putative PIN1 sites instead of 5 PXXPs and 1 PIN1 site in the human PRD); in the DNA-binding domain and the C-terminal part of the protein, numbering is lower by 3 amino acids in murine compared with human (mouse serine 389 is functionally equivalent to human serine 392); the C-terminal regulatory domain (CTD) of mouse p53 contains 7 lysines, instead of the 6 in human p53. Residues that are subject to stress-induced modifications and that have been targeted at the mouse p53 locus are shown. Below the protein are shown other targeted mutations, which provided valuable information on p53 function, but did not precisely target residues modified by stress. For several point mutations, abbreviated names are mentioned (for example, QS instead of L25Q,W26S). AMPK, adenosine monophosphate-activated protein kinase; ATM, ataxia telangectasia mutated; ATR, ataxia telangectasia and Rad3-related protein; AurK, Aurora kinase A; CAK, CDK-activating kinase; CDK, cyclin-dependent kinase; CHK, checkpoint kinase; CK, casein kinase; CSNK, cop-9 signalosome associated kinase complex; DNAPK, DNA-dependent protein kinase; ERK, extracellular signal-regulated kinase; GSK3, glycogen synthase kinase 3; HIPK2, homeodomain-interacting protein kinase 2; JNK,

c-Jun NH2-terminal kinase; MAPKAPK2, mitogenactivated protein kinase-activated protein kinase 2; p38, p38 kinase; PCAF, p300/CBP associated factor; PKC, protein kinase C; PKR, double stranded RNA-activated kinase; PLK3, pol-like kinase 3; RSK2, ribosomal S6 kinase 2; SET9, SET9 methyltransferase; SMYD2, SET/MYND domain-containing methyltransferase 2; SUMO, small ubiquitin-like modifier 1; TAF1, TATA-binding protein– associated factor 1; VRK1, vaccinia-related kinase 1.

1.7.2 Lysine modification

p53 C-terminal lysines are modified by ubiquitylation, acetylation, sumoylation, neddylation and methylation (FIG. 4a). Neddylation seems to inhibit transactivation, whereas sumoylation can positively or negatively affect p53 function (Bode et al. 2004). Recently, p53 sumoylation was proposed to induce senescence in normal human fibroblasts but apoptosis in RB (retinoblastoma 1)-deficient cells (Bischof et al.2006). Modifications of lysine 320 were proposed to promote cell-cycle arrest, rather than apoptosis (Di Stefano et al. 2005; Knights et al. 2006; LeCam et al. 2007). Unlike lysines 372, 373, 381 and 382, which are acetylated by p300 and ubiquitylated by MDM2 (Bode et al. 2004), lysine 320 is acetylated by the p300 and CBP associated factor (PCAF) (Di Stefano et al. 2005; Knights et al. 2006; Sakaguchi et al. 1998) and ubiquitylated by E4F1 (LeCam et al. 2007)). The E4F1-mediated lysine 48-like oligo-ubiquitylation has been proposed to induce cell-cycle arrest rather than promote p53 degradation (LeCam et al. 2007). Methylation at nearby lysines might also have dramatically different effects: lysine 372 methylation by SET9 stabilizes p53 (Chuikov et al. 2004), whereas the methylation of lysine 370 by SMYD2 destabilizes it. One interpretation of the increasing variety and complexity of p53 modifications at serines, threonines and lysines (FIG. 4a, b) is that the elegant model of p53 regulation.

1.8 Head and Neck Cancer

Head and neck cancer (HNC) accounts for about 5% of all cancers with >500 000 cases diagnosed worldwide and >100 000 in Europe each year. The majority of HNC in the Western world is of squamous cell origin (90% head and neck squamous cell carcinoma, HNSCC) and present with locally or regionally advanced disease (Rogers et al. 2005). HNSCC represents a group of treatment-refractory malignancies derived from cells within the basal epithelia of the aerodigestive mucosa (Forastiere et al. 2001). The most important risk factors are tobacco and alcohol (Decker et al. 1982; Jacobs et al. 1990; Winn et al. 1991; Falk et al. 1989; Nam et al. 1992). Alcohol potentiates tobacco-related carcinogenesis and is also an independent risk factor. Epidemiologic studies suggest a strong association between smokeless tobacco and oral carcinogenesis (Winn et al. 1981; Winn 1992). The use of marijuana is also of concern (Jacobs et al. 1990). Occupational risk factors include nickel refining, woodworking, and exposure to textile fibers (Decker et al. 1982; Jacobs et al. 1990; Brown et al. 1988; Muscat et al. 1992). An association of laryngeal

cancer with exposure to asbestos remains controversial (Jacobs et al. 1990; Muscat et al. 1992). Dietary factors may also play a part (Jacobs et al. 1990; Franceschi et al. 1990). Epidemiologic data suggest a protective role of dietary carotenoids (Peto et al. 1981) and an inverse association between the consumption of fruits and vegetables and the incidence of head and neck cancer (Peto et al. 1981; McLaughlin et al. 1988). The specific protective components in these foods remain to be identified. Increasing evidence suggests that viruses contribute to the cause of head and neck cancer (Shillitoe et al. 1984; Watts et al. 1991; Henle et al. 1976). DNA from human papillomavirus has been detected in cancerous tissue from the head and neck, (Watts et al. 1991), and infection with the Epstein-Barr virus is associated with nasopharyngeal cancer, a rare form of cancer in the United States but a common form in some North African and Asian countries. Information on the genetic basis of a possible multistep process of carcinogenesis is accumulating. Deletions of chromosome 3p (Heo et al. 1989; Sacks et al. 1988; Latif et al. 1992) and other nonrandom deletions and rearrangements of chromosomes have been identified in patients with head and neck cancer (Jin et al. 1988; Carey et al. 1989). Molecular changes include amplification and over-expression of the receptor for epidermal growth factor (Kamata et al. 1986; Ishitoya et al. 1989; Weichselbaum et al. 1989) and amplification of int-2, bcl-1, and other oncogenes (Zhou et al. 1988; Somers et al. 1990). In addition, p53 mutations have been described, and their occurrence may relate to the prognosis (Brachman et al. 1992; Gusterson et al. 1991). The finding of frequent deletions of chromosome 18q in patients with head and neck cancers suggests that other tumor-suppressor genes may also be affected (Cowan et al. 1992; Kelker et al. 1992).

1.8.1 Disabling p53 family network in head and neck cancer

Like other solid tumors, the p53 pathway is disabled by several mechanism in Head and neck squamous cell carcinomas (HNSCC). Early studies using immunocytochemisty suggested that p53 alterations are an early event in some HNSCC. In contrast, analysis of serial oral lesions biopsied from individual patients known to have progressed to oral cancer detected mutations in only 1/12 dysplasias, but in 6/8 carcinomas. These results suggest that mutation is a relatively late event in carcinogenesis in the oral cavity, wherein it is associated with acquisition of the invasive phenotype (Shahnavas et al. 2000). Another, common molecular abnormality observed in these tumors is over-expression of the p53 family member p63. Numerous studies have documented increased p63 expression in up to 80% of primary HNSCC tumors, and its overexpression is also commonly observed in other squamous epithelial malignancies, including lung and esophagus (Hu et al. 2002; Massion et al. 2003; Sniezek et al. 2004 and Weber et al. 2002). *p63* maps to chromosome 3q, and human squamous cell carcinomas (SCCs) frequently exhibit genomic amplification at 3q (Bjorkqvist et al. 1998). The relevance of these observations is supported by

data showing that increased *p63* mRNA levels correlate with increased *p63* gene copy number in squamous cell carcinomas (SCCs) of the lung and head and neck (Hibi et al. 2000). In some cases, overexpression of p63 is likely to involve mechanisms independent of genomic amplification (Redon et al. 2001). In either case, it is apparent that overexpression of p63 is one of the most common molecular abnormalities identified in HNSCC. Nevertheless, the precise contribution of p63 overexpression to HNSCC remains undefined. The essential function of p63 in the epithelium is evidenced by the phenotype of *p63* null mice, which exhibit profound developmental failure of the epidermis and oral epithelium, as well as abnormalities of limb, prostate, and mammary development (Mills et al. 1999 and Yang et al. 1999). Expression from two distinct *p63* promoters produces protein isoforms that either contain or lack the N-terminal transactivation domain (TAp63 and ΔNp63, respectively). Differential mRNA splicing also gives rise to multiple C-terminal variants (Yang et al. 1998). In both normal epithelia and in HNSCC cells, the predominant p63 isoform expressed is ΔNp63α (Parsa et al. 1999 and Yang et al. 1998). While few bona fide transcriptional target genes of p63 have been identified, ΔNp63α is known to function as a transcriptional repressor of endogenous cell cycle inhibitors including $p21^{CIP1}$, implying a contribution by p63 to cellular proliferation (Westfall et al. 2003). Other studies have proposed roles for p63 in cell survival, cellular differentiation, and morphogenesis (King et al. 2003; Mills et al. 1999 and Yang et al. 1999). In addition to its role in normal epithelia, ΔNp63α has been hypothesized to contribute to tumorigenesis based on its ability to inhibit p53-dependent transactivation in vitro following ectopic expression of these proteins (King et al. 2003 and Yang et al. 1998). Such observations supported a model whereby overexpression of p63 might inactivate p53, therefore abrogating the requirement for its loss during tumorigenesis. Whether these findings reflect an endogenous function of p63 remains uncertain, since no consistent correlation has been proven between p53 mutation and p63 overexpression in SCCs (Choi et al. 2002, Hibi et al. 2000, Sniezek et al. 2004 and Weber et al. 2002). Similarly, although ectopic ΔNp63α expression can block p73-dependent reporter transactivation, and p73 and p63 associate in cotransfection assays (Chan et al. 2004), it remains to be determined whether endogenous p63 exhibits either a physical or functional interaction with p73 in tumor cells. Indeed, it has been proposed that p63 promotes oncogenesis in HNSCC cells by a distinct mechanism-involving enhancement of β-catenin-dependent transcription (Patturajan et al. 2002). Thus, the contribution of any potential interaction between p63 and other p53 family members in SCC remains to be defined. Other than direct intrinsic biological factors disabling p53 net work in HNSCC, extrinsic a cellur viruces (eg. Human paipillomavirus, HPV) are well known.

1.8.2 Implications of human papillomavirus in head and neck cancers

Certain mucosal HPV types, termed high risk HPVs, play a role in the pathogenesis of head and neck squamous cell carcinomas (HNSCC) (Schwartz et al. 1998; Gillison et al. 2000; Andl et al. 1998; Wiest et al. 2002; van Houten et al. 2001; Hafkamp et al. 2003; Mork et al. 2001). High-risk, oncogenic types (eg, HPV16, -18, -31, -33, -35) are defined by their strong epidemiologic association with cervical cancer (Munoz et al. 2003). The prototypic high-risk types -16 and -18 are capable of transforming epithelial cells derived from both the genital and upper respiratory tracts (McDougall 1994). The transforming potential of high risk HPVs is largely a result of the function of two viral oncoproteins, E6 and E7, which functionally inactivate two human tumor-suppressor proteins, p53 and pRb, respectively (Munger et al. 2002). Expression of high-risk HPV E6 and E7 results in cellular proliferation, loss of cell cycle regulation, impaired cellular differentiation, increased frequency of spontaneous and mutagen-induced mutations, and chromosomal instability (Munger et al. 2002). Emerging lines of evidence support the role of another group of HPV in human carcinogenesis: the cutaneous HPVs are classified into different genera, of which the genus *Beta-papillomavirus* contains 23 different fully characterized HPV types (previously designated "EV" types). Specific cutaneous HPV types have been associated with squamous cell carcinoma (SCC) of the skin of individuals with the rare hereditary disease *Epidermodysplasiaverruciformis* (EV) (Jablonska et al. 1994), but such association has not been reported for SCC in the general population.

1.8.2.1 HPV DNA presence and expression in HNSCC cancers

Infection by a high-risk HPV type is now known to be associated with the development of cervical cancer. In contrast to cervical cancer, in HNSCC, HPV appears to play a carcinogenic role for only a subset (Schwartz et al. 1998; Gillison et al. 2000; Hafkamp et al. 2003; Balz et al. 2003; Pintos et al. 1999; Snijders et al. 1996; Brandwein et al. 1994; Paz et al. 1997; Fouret et al. 1997; Ritchie et al. 2003; Haraf et al. 1996). In a recent meta-analysis, HPV genomic DNA was detected in approximately 26% of all HNSCC by sensitive polymerase chain reaction (PCR) -based methods (Kreimer et al. 2005). However, data are most strong and consistent for HPV presence in oropharyngeal cancers. In the majority of studies, 50% or more of oropharyngeal tumors contained the HPV genome (Ritchie et al. 2003; Haraf et al. 1996; Strome et al. 2002). In a recent multinational study conducted by the International Agency for Research on Cancer (IARC), only18% of oropharyngeal tumors were HPV positive, indicating that this proportion likely varies by geography (Herrero et al. 2003). Regardless of the study population, high-risk HPV16 accounts for the overwhelming majority (90% to 95%) of HPV-positive tumors, whereas other high-risk types -31, -33, and -35 account for the minority (Kreimer et al. 2005; Herrero et al. 2003). For oropharyngeal tumors, viral HPV DNA has been specifically localized to tumor cell nuclei,

(Gillison et al. 2000; Hafkamp et al. 2003) is frequently integrated (Gillison et al. 2000; Wiest et al. 2002; Hafkamp et al. 2003; Steenbergen et al. 1995) and is transcriptionally active (Gillison et al. 2000; Wiest et al. 2002; Ke et al. 1999). Furthermore, HPV is present in high copy number in tumor cell nuclei of in situ, invasive, and metastatic disease and absent in adjacent normal tissue (Begum et al. 2003). These data indicate that HPV infection is specific to tumor cell nuclei and that infection precedes histopathologic progression of the tumor. Equivalent data are not available for non-oropharyngeal tumors. For instance, HPV has not been shown to have an etiologic association with oral tongue cancers diagnosed at any age. Although HPV can infect the epithelium of the upper aerodigestive tract in general, the tonsil appears uniquely susceptible to transformation by the virus. As for the transformation zone of the cervix, the reason for this anatomic site specificity for transformation is unknown. A role for HPV in oropharyngeal tumors is further substantiated by distinct molecular genetic alterations in HPV-positive versus HPV negative tumors. As for many cancers, inactivation of the p53 and pRb pathways is a common event in the molecular progression of HNSCC. However, inactivation occurs by different mechanisms in HPV positive and -negative tumors. In HPV-positive HNSCC, genetic alterations are reflective of viral oncogene function. For instance, HPV-positive tumors tend to have wild-type p53, because p53 is functionally inactivated by viral E6 oncoprotein (Brachman et al. 1992; Chiba et al. 1996). By contrast, HPV-negative tumors have specific p53 mutations demonstrated to be induced by carcinogens in tobacco smoke (Gillison et al. 2000; Wiest et al. 2002; Hafkamp et al. 2003; Brachman et al. 1992; Chiba et al. 1996). As another example, pRb function is inactivated by viral E7 protein in the HPV-positive tumor, but in HPV-negative tumors, the pRb pathway is altered by other mechanisms, including amplification of cyclin D and inactivation of p16INK4a (Andl et al. 1998; Wiest et al. 2002; Hafkamp et al. 2003; Begum et al. 2003). More complex differences in regions of chromosomal loss and gain have been demonstrated in HPV-positive versus -negative tumors through techniques such as comparative genomic hybridization (Smeets et al. 2005) and microsatellite analysis (Braakhuis et al. 2004).

1.9 EGFR and solid tumors

The Epidermal growth factor receptor (EGFR also known as ErbB1 or HER) was the first receptor identified of the ErbB family of receptors (Sato et al. 1983). Since then, the ErbB family proteins have increased to four, including EGFR-1 itself (HER- 1, ErbB1), HER-2 (ErbB2), HER-3 (ErbB3) and human HER-4 (ErbB-4) (Carpenter et al. 1987). The EGFR, a 170 kDa glycoprotein, consisting of an extra-cellular domain, a transmembrane region and an intracellular domain with tyrosine kinase function, responds to numerous ligands, such as transforming growth factor alpha (TGFa), betacellulin, amphiregulin, epiregulin, EGF and heparinbinding EGF (Rogers et al, 2005).

In the early 1980s, the discovery that alterations in the EGFR signaling pathway contribute to malignant transformation was initially made in studies of oncogenic viruses that demonstrated that the EGFR is the cellular homolog of the avian erythroblastosis virus v-erbB oncogene. This encodes a truncated EGF receptor (or closely related protein) lacking the external EGF binding domain but retaining the transmembrane domain and the domain involved in stimulating cell proliferation (De Larco et al. 1980; Downward et al. 1984; Roskoski et al. 2004). Subsequently, it was discovered that activation of the EGFR signaling pathway mediates the malignant transformation of virus-infected cells (Miller et al. 1995). Furthermore, over-expression of members of the ErbB family, EGFR and ErbB2, has been demonstrated to induce malignant transformation in NIH-3T3 cells (Di Fiore et al. 1987). Malignant transformation as a consequence of EGFR dysregulation can occur in humans by different mechanisms, including receptor over-expression, activating mutations, alterations in the dimerisation process, activation of autocrine growth factor loops, limited or enhanced endocytosis of activated receptor, deficiency of specific phosphatases deactivating the phosphorylated EGFR tyrosine residues, and limited turnover. EGFR gene over-expression, without gene amplification, and EGFR activation, by TGF-α in an autocrine loop, are two of the main frequent mechanisms implicated in cancer development and progression (Athale et al. 2006; El-Obeid et al. 2002). Moreover, it has been reported that EGFR is mutated in some cancers and this is particularly prevalent in glioblastoma (Collins et al. 1994). While multiple types of mutations have been found, one specific mutation is far and away the most common. This mutation results in the expression of a truncated EGFR designated EGFRvIII (also referred to as del2-7 EGFR or ΔEGFR in the literature) (Yamazaki et al. 1990; Wong et al. 1992). In this mutation, the information coded for by exons 2 through 7 of the 26-exon EGFR gene is lost. Transcription of the mutated gene gives rise to an mRNA with an 801 base pair deletion. This deletion is in-frame, and a new glycine codon is formed at the fusion junction. Translation of this mRNA in turn gives rise to an EGFR in which amino acids 6–273 are replaced by a single glycine residue, resulting in a 145-kDa glycoprotein with constitutive, ligand-independent activation of the receptor's tyrosine kinase activity (Chu et al. 1997; Moscatello et al. 1998). Other than this well known truncated EGFR variant, single nucleotide somatic missense mutations, as well as small in-frame deletions and insertions have recently been reported in the EGFR tyrosine kinase domain. These somatic mutations (L858 and del747–752) also appear to be oncogenic in nature, and may provide insights into the potential role of altered EGFR signaling pathways in tumorigenesis (Jiang et al. 2005; Greulich et al. 2005). Many cell types normally and widely express EGFR, including those of epithelial and mesenchymal lineages (Wells 1999). The variability in over-expression or dysregulation of EGFR has been also described for human malignancies (Nicholson et al. 2001). Epidemiological evidence accumulated over the last 22 years in human tumors supports the notion that aberrant EGFR expression and

signalling contribute to the development of multiple epithelial malignancies in humans including squamous carcinomas of the head and neck and breast cancer among others (Ibrahim et al. 1997).

1.9.1 Epidermal Growth Factor Receptor Signaling in HNSCC

In normal cells, the expression of EGFR ranges from 40,000 to 100,000 receptors per cell (Carpenter et al. 1979). In SCCHN, EGFR and its ligand, TGF-α, are over-expressed in 80-90% of cases; the corresponding magnitudes of increase are 1.7-fold (P=0.005) and 1.9-fold (P=0.006) respectively, when compared to controls (Grandis et al. 1998).The nature of the protein overexpression is thought to result from enhanced transcription, with no apparent change in mRNA stability; gene amplification has been observed less frequently. EGFR overexpression is an early event in HNSCC carcinogenesis; it is already present in "healthy" mucosa (field cancerization) from cancer patients, when compared to healthy controls; this overexpression will increase steadily in parallel to observed histological abnormalities, from hyperplasia to invasive carcinoma, through dysplasia and in situ carcinoma (Rubin Grandis et al. 1996). Dysregulated p53, polymorphisms in dinucleotide repeats in intron 1 of the EGFR gene and EGFR amplification can all lead to increased EGFR mRNA synthesis. However, EGFR gene amplification was only observed in seven out of 33 patients with SCCHN and did not correlate with EGFR protein overexpression, suggesting that gene amplification is not pathogenetically involved in EFGR protein overexpression (Mrhalova et al. 2005). Furthermore, overexpression of cortactin may inhibit ligand-induced EGFR downregulation. Interestingly, tobaccos smoke increases EGFR ligand levels (e.g. amphiregulin and TGF-α) culminating in EGFR activation and increased levels of cyclooxygenase 2 and prostaglandin E2, which can transactivate EGFR (Kalyankrishna and Grandis 2006). Recently, three identical in-frame deletions in exon 19 (E746_A750del) of the EGFR gene were reported in three out of 41 (7.3%) Korean SCCHN cases (Lee et al. 2005). In contrast, EGFR kinase domain mutations were rare among US (zero out of 65) or European (one out of 100) SCCHN cases (Cohen et al. 2005; Loeffler-Ragg et al. 2006). Interestingly, one gefitinib-responsive SCCHN patient harboured a heterozygous mutation within ErbB2 (V773A) (Cohen et al. 2005). ErbB2 heterodimerises with EGFR and ErbB2 mutations have recently been reported within a subset of non-small cell lung cancer (NSCLC). Epidermal growth factor receptor vIII, a deletion of exons 2–7 resulting in a truncated extracellular domain and constitutive tyrosine kinase activation, has been reported in SCCHN (42%); (Sok et al. 2006). Downstream effects of EGFR activation after receptor dimerisation, internalisation and autophosphorylation are mediated through several signal transduction pathways involving the RAS/ MAP kinase, the phosphatidylinositol 3-kinase (PI-3K)/Akt, the PLCg and the JAK-STAT pathways (Rogers et al. 2005; Kalyankrishna and Grandis 2006). Although the main autophosphorylation sites in ErbB receptors recruit

extensively overlapping molecules to the active receptors, preferential modulation of signaling pathways seems to occur (e.g. EGFRs with kinase-domain mutations preferentially activate the pro-survival PI-3K/AKT pathway and the STAT pathway). Downstream effectors of EGFR (e.g. ERK-1/2, AKT, STAT-3/5) are activated in SCCHN (Kalyankrishna and Grandis 2006). Furthermore, EGFR can be activated by other receptor tyrosine kinases including insulin-like growth factor-1 receptor, adhesion molecules (e.g. E-cadherin and integrins) and G-protein-coupled receptors (GPCR).

1.10 Head and Neck Cancer Therapy

Head and Neck cancer patients with early-stage disease are treated with surgery and/or radiotherapy and nearly 80% are cured. Chemotherapy added to locoregional treatment provides a demonstrated survival benefit in nonmetastatic SCCHN (Pignon et al. 2000). However, despite combined treatment approaches (surgery and radiation/chemoradiation therapy) most patients with resectable advanced disease develop local or regional recurrences (50–60%), metastatic disease (20%) or secondary primaries. Patients with unresectable advanced disease have a 5-year survival of 10% and recurrent/metastatic cases have a median survival of approximately 6–9 months, which has not changed significantly for 30 years. Several therapeutic options are available for these patients, including irradiation, salvage surgery, palliative chemotherapy or best supportive care for patients with low performance status. The most commonly used agents are cisplatin or carboplatin, often in combination with taxanes or 5-fluorouracil. Response rates (RR) to first-line platinum-based chemotherapy are only 30%. In recurrent/ metastatic SCCHN, survival benefits of 10 weeks may be expected (Morton et al. 1985; Browman and Cronin 1994). Although several combinations of classical chemotherapeutics have increased RR, improved survival has not been observed. Options and RR of patients refractory to platinum-based therapies are generally very poor. Therefore, there is clearly an unmet therapeutic need for new active, less toxic agents for SCCHN treatment. In recent years, the field of cancer therapy has witnessed the emergence of novel targeted strategies that inhibit specific cancer pathways and key molecules in tumor growth and progression. Among them, one class of compounds that has shown great progress is those targeting tyrosine kinases (TKs), their ligands, and signal transducers. Over 20 years ago, Mendelsohn et al proposed that the EGFR was a target for cancer therapy (Mendelsohn et al. 2003; Kawamoto et al. 1983; Sato et al. 1983). With two classes of anti-EGFR agents with established clinical activity in cancer, this hypothesis has now been confirmed. These are monoclonal antibodies directed at the extracellular domain of the receptor and small molecule, adenosine triphosphate (ATP) -competitive inhibitors of the receptor's TK (Mendelsohn et al. 2003).

1.10.1 Targeting EGFR in HNSCC

Two principal methods to inhibit EGFR have been identified and are being used in clinical trials, small molecule inhibitors of EGFR and antibodies to EGFR. Two small molecule inhibitors, ZD1839 and erlotinib HCl, OSI-774, and one antibody, C225 (IMC-C225) have been evaluated in SCCHN. Each of these inhibitors has unique mechanisms of action and pharmacodynamics. The small molecule inhibitors can be delivered orally and specifically inhibit the enzymatic function of the EGFR. Antibodies can perform diverse functions, including blocking of ligand/receptor binding, immune functions, and removal of target from the cell surface by ingestion or secretion. C225 prevents binding of activating cytokines to the EGFR and causes EGFR movement into the cytoplasm, where it is sequestered and unavailable. Inhibition of EGFR, either by antibodies or small molecule inhibitors, can cause tumor regression in animal models of human tumors; however, slowing or stabilization is a more common effect. More robust anti-tumor activity is observed when the inhibitors are combined with standard chemotherapy agents and radiotherapy. This is because EGFR activation seems to protect malignant cells from chemotherapy- and radiotherapy-induced cell death. Blocking EGFR activation enhances tumor specific destruction by standard chemotherapy agents and radiotherapy. ZD1839 has been shown to inhibit EGFR activation and downstream signaling in normal skin at doses acceptable for both primary and combination therapy. Phase 1 testing with ZD1839 suggests activity in SCCHN (Albanell et al. 2002; Albanell et al. 2001; Ranson et al. 2002). Ultimately, the role of ZD1839 and C225 and other inhibitors in the treatment of primary or recurrent SCCHN will depend on the outcome of combination trials that explore the potential interactions with many different standard treatments, including taxanes, anthracyclines, biologics, and radiotherapy techniques. It should be remembered that it has taken many years to explore the potential of drugs like interferon or 5-fluorouracil, and to understand how to use them optimally in patients. We should expect that it would take some time to understand how to use this whole new family of TKIs including erlotinib, and newer agents such as the ErbB family TKI, CI-1033.

1.10.1.1 EGFR TKIs

Both ZD1839 (Gefitinib) and OSI-774 (formerly known as CP-358-774, Erlotinib) have FDA approval for treatment of locally advanced or metastatic NSCLC since May 2003 and November 2004, respectively. Three orally active EGFR inhibitors have been tested in clinical trials in recurrent/metastatic SCCHN or in combination with radiotherapy in locoregionally advanced SCCHN. Gefitinib (IressaR, AstraZeneca Pharmaceuticals, London, UK) impeded in vitro and in vivo growth of cell lines that express high, intermediate or low levels of EGFR and high levels of HER-2. Furthermore, gefitinib has additive or synergistic properties in combination with cisplatin, carboplatin, paclitaxel, taxanes, doxorubicin and radiotherapy. A phase II trial of 500 mg

gefitinib in 52 patients with recurrent/metastatic HNSCC reported an RR of 10.6% and a DCR of 53% (Cohen et al. 2003). Squamous cell carcinomas of the head and neck responses to gefitinib or erlotinib seem not to be linked to EGFR kinase mutational status, as these mutations are rare in this disease (Cohen et al. 2005). Recently, a phase I study in SCCHN reported that gefitinib (250/500 mg q.d.) in combination with celecoxib (200/400 mg b.i.d.) is very well tolerated in patients with incurable SCCHN (Wirth et al. 2005).

1.10.2 p53 family proteins influence in Head and Neck Cancer Therapy

Historically, the p53 gene was considered the most important determinant of response to radiotherapy or chemotherapy. Many studies conducted during 1980– 1990 that aimed to correlate the response to treatment with p53 gene expression produced conflicting results (Partridge et al. 2005). It is now known that apoptosis is a fundamental mechanism of cell death following treatment with cytotoxic agents. Wild-type p53 may enhance chemosensitivity by promoting apoptosis, and some studies have suggested that the presence of a p53 mutation is associated with a lower response to chemotherapy (Cabelguenne et al. 2000; Cutilli et al. 1998 and Fouret et al. 2002). Contradictory studies have also been reported, in that tumours that over-expressed p53 were more responsive to cisplatin than those that did not express (Honeycutt et al. 2004). Recent investigations have shown that some of this variation in chemosensitivity may be due to a common sequence polymorphism of the p53 gene that results in either proline or arginine at amino acid position 72. This Arg 72 polymorphism is associated with a good response to chemotherapy and radiotherapy in clinical trials (Bergamaschi et al. 2003). This effect is not due to p53 acting alone, as TA p73 is also induced by many chemotherapeutic agents and reinforces the apoptotic response to chemotherapy. The accumulating evidence converges on the view that p73 plays a significant role in curative anti-cancer therapy. Similar to p53, activated p73 mediates a cellular response to radio and chemotherapy, including -irradiation and treatment with cisplatin, doxorubicin, camptothecin, etoposide, bleomycin, mitoxantrone, taxol, and the cytosine analogues gemcitabine, Ara-C and T-ara-C (Agami et al., 1999; Costanzo et al., 2002; Gong et al., 1999; Thottassery et al., 2006; Vayssade et al., 2005). For instance, cisplatin, which several groups have found to activate and stabilize the p73 protein, does not induce p73 mRNA. Doxorubicin and taxol can induce p73 mRNA and protein at the same time. In contrast, -irradiation increases the p73 protein activity without affecting the protein or mRNA levels (Agami et al., 1999; Hamer et al., 2001). However, other studies reported accumulation of p73 following _-irradiation (Dai et al., 2007; Lin et al., 2004). It is worth noting that p73 protein accumulation can be transient and depend on a treatment regiment (Irwin et al., 2003; Lin et al., 2004). TAp63 isoforms are also induced by various genotoxic agents, although their effect appears to be weaker than that of p53 and TAp73. Similarly, TAp63 can be a determinant of chemotherapeutic efficacy in some

cell types as inhibition of endogenous TAp63 with siRNA leads to a decreased chemosensitivity (Gressner et al., 2005). Combined loss of p73 and p63 results in the failure of mouse embryonic fibroblasts containing p53 to undergo apoptosis in response to DNA damage (Flores et al., 2002). ΔN isoforms of p73 and p63 as well as mutant p53 are also involved in cross regulation. Upon treatment with doxorubicin, ΔNp73 and mutant p53 associate with the ΔNp63 promoter and induce the ΔNp63 transcription through proximal CCAAT boxes. This may have an anti-apoptotic effect and induce chemo-resistance (Lanza et al., 2006). In head and neck tumors, high ΔNp63 protein levels correlate with a favorable response to platinum-based therapy (Leong et al., 2007; Zangen et al., 2005). It suggests that the dominant negative concept cannot explain all complexity of interactions attributed to ΔN isoforms. These interactions are determined by cellular context and should be analyzed through a prism of multiple interactions within and beyond the p53 family. Moreover, how p53 family network influence to newly developed molecular drugs, e.g. Gefitinib yet to be characterized.

2. AIMS OF THE STUDY

p53 family protein netwok in cancer biology has atained much importance because of its redundant functions in normal versus cancer cells as well as this redundancy influences to different modes of anti-cancer therapy. In almost all cancers, including HNSCC development, at some stage of signal transduction pathway, p53 network has been disabled and that causes the immortalization potential of a single cell to advanse to an metastatic carcinoma. Recently, the discover of p53 family p63 and p73 proteins similarity with p53 indicates that it has some more redundant functions related with p53 as their truncated isoforms can act as a dominant negative effect on p53 and their parental proteins p63 and p73 itself. Moreover, detailed analysis of p53 functional role indicates a positive role in normal cell survival but it is unknown if this cell survival function has an over advantage, in cancer cells expressing wild type p53 protein. Accordingly, studies from our lab have showed that HPV38 mediated immortalization of human keratinocytes accumulate wild type p53 hyperphosphorylated at serine15 and 392 which leads to the overexpression of ΔNp73 isoform and likely assists cell survival. Therefore, we have tried to molecularly characterize accumulated wild type p53 properties and functions in HPV positive and negative cancer cell lines as using HNSCC cell lines as a study model. Along with our molecular cancer biology studies, we aim to identify how p53 family network influences to new molecular based therapeutic strategies. One of the more promising is the utilisation of tyrosine kinase (TK) inhibitors, targeting epidermal growth factor receptor (EGFR) in HNSCC. Therefore: as a prelimenary step, we aim to characterise basic features of newly established HNSCC cell lines; including, morphological features, cell cycle and ploidy analysis, doubling time and HPV detection. Moreover, we have aimed as follows;

- The first aim of this study was to understand in detail of p53 family network in these cell lines that includes, p53 mutation analysis, expression and its cellular localization. And other p53 family proteins, p63 and p73 and their main isoforms expressions and dominant negative effects in HNSCC.
- The second aim was to understand properties and functions of accumulated wild type p53 role in HPV positive and negative cancer cell lines
- Characterization of EGFR family receptors and its major down stream effectors status in this large panel of cell lines
- Specific EGFR inhibitor, gefitinib effectiveness and mode of action in this large panel of HNSCC cell lines
- Comparison between the targets analyzed and gefitinib effectiveness
- Chemoresistance in head and neck cancer cell lines

3. MATERIALS AND METHODS

Reagents

3.1.a. Chemical reagents

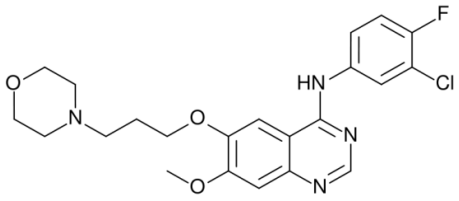
General laboratory reagents were obtained from a variety of suppliers and were of analytical grade or purer. Sources of reagents are always stated whenever they are used in the method description. The composition of the various solutions used is always stated whenever they are used in the methods descriptions.

Acrylamide (Gibco)
Agarose (Gibco)
Ampicillin (Sigma)
BCA Protein assay reagents (Pierce)
Calf serum (Sigma)
ECL detection reagent (Amersham)
Foetal calf serum (Life Technologies)
Ligase buffer (Boehringer Mannheim)
DNA Mass loading dye (Fermentas)
3MM Gel blotting paper (Schleicher and Schuell)
Polyscreen PVDF transfer membrane (NEN Life Science Products)
Powder milk (Sigma)
Trypsine/EDTA 0.125% (Gibco-Invitrogen)
PBS – Phosphate Buffered Saline (Cambrex)
DMSO – Dimethyl sulfoxide (Merck)
DMEM – Dulbecco's modified Eagle's medium (Sigma)
L-Glutamine (Gibco-Invitrogen)
Pen-strep (Gibco-Invitrogen)

3.1.b Compounds

Gefitinib (originally coded ZD1839), *N*-(3-chloro-4-fluoro-phenyl)-7-methoxy- 6-(3-morpholin-4-ylpropoxy)quinazolin-4-amine, was kindly provided by Astra Zeneca (Pharmaceuticals, Macclesfield, UK).

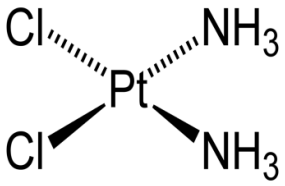
Figure 5 : Chemical structure of ZD1839



Stock solutions were prepared at 20mM in 100% dimethylsulfoxide (DMSO) and stored in aliquots at -20°C. From stock solutions, required concentration prepaired by diluting with culture media before use.

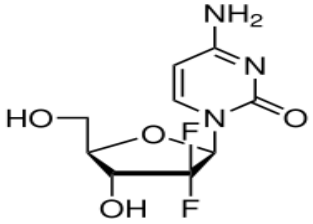
Cisplatin, cisplatinum or *cis*-diamminedichloridoplatinum(II) (CDDP), was purchased from Sigma Chemical Co. (St Louis, MO)

Figure 6: Chemical structure of Cisplatin



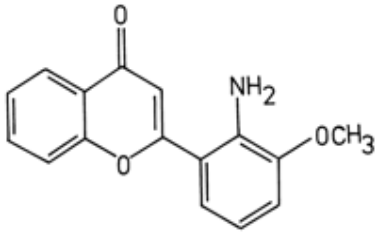
Gemcitabine, 4-amino-1-[3,3-difluoro-4-hydroxy-5- (hydroxymethyl) tetrahydrofuran-2-yl]- 1H-pyrimidin- 2-one, was purchased from Eli Lilly (Indianapolis, IN).

Figure 7: Chemical structure of Gemcitabine



PD 098059, was purchased from Cell Signaling Technology, Inc. (Beverly, MA).

Figure 8: Chemical structure of PD 098059



PD 098059 was dissolved in dimethyl sulfoxide (Me2SO) to give a concentration of 50 mM, stored in aliquots at -80 °C, and diluted in aqueous buffers to <100 µM immediately prior to use. For studies *in vitro*, PD 098059 or the equivalent amount of (Me2SO) carried over with the drug was incubated with target cells for 48 hours.

3.1.c. Biological materials

Kits

Taq DNA Polymerase recombinant (Invitrogen)
dNTPs set PCR grade (Invitrogen)
Absolutely RNA miniprep kit (Stratagene)
First strand cDNA synthesis kit (Fermentas MBI)

Markers

DNA size markers
λDNA/HindIII (Fermentas)
Mass Ruler DNA Ladder Mix (Fermentas)

Protein size marker

RAINBOW molecular weight standard prestained marker (Amersham)

3.2 DNA constructs

The following retroviral vectors were used: pBabe (described by Caldeira et al, 2003), and pRetroSuper (Screeninc, Amsterdam, The Netherlands). ΔNp73a complementary DNA, kindly provided by Ute Moll (Stony Brook, US), was cloned in pBabe. p53 expression was silenced using the oligonucleotides listed in Table 1.

Table 1

Primer	Sequence
siRNA p53	5_-CAAUGGUUCACUGAAGACC-3_
siRNA scranble	5_-CAUAGAUUACCGUGAGCAC-3_

Table 1. Sequences of different primers used for gene silencing.

Table 2

Genes/promoters	Primer sequence
Human GAPDH (RT PCR)	F. 5_-AAGGTGGTGAAGCAGGCGT-3_ R. 5_-GAGGAGTGGGTGTCGCTGTT-3_
Human-ΔNp73	F. 5'-AACCATGCTGTACGTCGGTGACCCC-3' R. 5'-GCGACATGGTGTCGAAGGTGG-3'
Human-ΔN'p73	F. 5'-TCGACCTTCCCCAGTCAAGC-3' R. 5'-TGGGACGAGGCATGGATCTG-3'
ΔNp63	F. 5'-TGC CCA GAC TCA ATT TAG TGA G-3' R. 5'-AGA GAG AGC ATC GAA GGT GGA G-3'
TAp63	F. 5'-GAC CTG AGT GAC CCC ATG TG-3' R. 5'-CGG GTG ATG GAG AGA GAG CA-3'
Human-TAp73	F. 5'-AACCAGACAGCACCTACTTCGACC-3' R. 5'-GCGACATGGTGTCGAAGGTGG-3'
Human SCO2	Forward 5'-GCA GCC TGT CTT CAT CAC TGT GGA CC-3' Reverse 5'-CCG CAC ACT GTC TGA GAT CTG CTC-3'
Human EGFR	
EXON 18	Fw 5' CAAATGAGCTGGCAAGTGCCGTGTC 3' Rv 5' GAGTTTCCCAAACACTCAGTGAAAC 3'
EXON 19	Fw 5' GCAATATCAGCCTTAGGTGCGGCT 3' Rv 5' CATAGAAAGTGAACATTTAGGATGTG 3'
EXON 20	Fw 5' CCATGAGTACGTATTTTGAAACTC 3' Rv 5' CATATCCCCATGGCAAACTCTTGC 3'
EXON 21	Fw 5' CTAACGTTCGCCAGCCATAAGTCC 3' Rv 5' GCTGCGAGCTCACCCAGAATGTCTGG 3'
EXON27	27 Fw 5' CAACCTACTAATCAGAACCAGCATC 3' Rv 5' CCTTCACTGTGTCTGCAAATCTGC 3'
EXON28a	Fw 5' CCTGTCATAAGTCTCCTTGTTGAG 3' Rv 5' GGTAGTCAGGGTTGTCCAGG 3'
EXON28b	Fw 5' CGAGTATCTCAACACTGTCCAGC 3' Rv 5' CTAATGCGGGCATGGTG 3'

Table 2. Sequences of the different primers used for PCR and RT- PCR analyses. The sequences of forward (F) and reverse (R) primers for gene listed in the table are shown.

3.3 Cell culture

3.3.1. Cultivation conditions

Used cell lines were cultivated in Dulbecco´s Modified Eagle Medium (DMEM). Before using the medium 100 U/ml Penicillin, 0.1 mg/ml Streptomycin and 2 mM Glutamine were added. To cultivate NIH/3T3 the medium was supplemented with 10 % calf serum (CS), whereas for all other cell lines 10 % foetal calf serum (FCS) was used. The serum was inactivated before use by a 45 min incubation period at 55°C. The cultivation was done in either, flasks, dishes or multi-well plates in an incubator producing a water saturated environment of 37°C and 5 % CO2 concentration. To reduce the risk of infections and contaminations when handling cells, work was always done under a laminar flow. Before adherent cells reached confluency they were trypsinised and split. For this, the culture medium was removed and the cells were washed twice with 37°C warm PBS and the appropriate amount of trypsin/EDTA solution (3 ml for a 175 cm2 flask) was added. The cells were incubated at 37°C and monitored under the microscope. As soon as over 80 % of the cells were floating, medium was added (10-20 ml for a 175 cm2 flask) and the cells were dispersed by pipetting up and down a few times. Counting of cells can be done at this stage (see below). The suspension of cells was then divided accordingly, pipetted into different culture vessels and filled up with medium. Phoenix, was grown in DMEM supplemented with 10% fetal calf serum.

Primary human keratinocytes were isolated from skin of adult individuals as previously described (Caldeira et al, 2003) and grown together with NIH 3T3 feeder layers in FAD medium containing 3 parts Ham's F12, 1 part DMEM, 5% fetal calf serum, insulin (5 g/ ml), epidermal growth factor (10 ng/ml), cholera toxin (8.4 ng/ml), adenine (24 g/ml), and hydrocortisone (0.4 g/ml). Feeder layers were prepared by irradiating NIH 3T3 (137Cs; 80 Gy).

3.3.2 Counting of cells

For the growth rate of cultured cells the initially added cell number is an important factor. Thinly seeded cells will grow only very slowly, whereas high initial number will mean cells have to be split very often. Hence, counting of cells is a good method to get the right cell density at the desired time point. A fast and efficient tool is the Neubauer counting chamber. This chamber consists of nine big squares, which have an area of 1 mm2. With a depth of 0.1 mm this gives a volume of 0.1 µl. The surface of the chamber and the cover slip should be cleaned with 70 % ethanol. The cover slip should be moistened slightly and pressed onto the counting chamber, until Newton rings appear between the chamber and cover slip. 10 µl of a homogenous suspension of cells can be applied to the corner of the cover slip. The suspension moves into the chamber by capillary force. Counting can subsequently be done under the microscope.

At least four big squares should be counted to calculate the average. The actual cell number per ml is calculated by multiplying the average of the four squares by 10000.

3.3.3 Freezing and thawing of cells

For freezing, eukaryotic cells were detached from the culture vessel as described in point 3.3.1. The cell suspension was transferred into a 15 ml sterile tube and centrifuged for 10 min at 1,000 g. The pelleted cells are carefully resuspended in 5 ml cold Cell Culture Freezing Medium and then pipetted as 1 ml aliquots into cryo vials. The vials were placed into a cryobox (Cryo 1°C Freezing Container, Nalgene) which was cooled by Isopropyl alcohol and stored for 24 h at -70°C. This gentle procedure allows the cells to freeze slowly (-1°C/min) leading to higher viability when thawing. After the 24 h period the cells were transferred to liquid nitrogen for long term storage. For the thawing of cells a cryo vial is placed for 5 min at 37°C and immediately resuspended in 10 ml pre-warmed medium. To remove the toxic DMSO included in the Cell Culture Freezing Medium the cells were centrifuged, resuspended in fresh medium and seeded into a culture vessel. After approx. 24 h the condition of the cells was checked and the medium was changed.

Cell Culture Freezing Medium: DMEM
20 % FCS or CS
7 % DMSO

3.4 In-vitro growth-inhibition assay

As our most cell lines are very slow in growth, cells were seeded at a density of 10,000-12,000 cells per well in 96-well plates and allowed to attach for 24 hours. Cells were then treated with various concentration of EGFR inhibitor (Iressa) (0.001 to 100μM) in the presence of 10% serum for 72 hours, at the end of which 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (5mg/ml) was added to each well. After an incubation of 1 hour, absorbance was read at 570nmol/L. Values for control cells were considered as 100% viability.

3.5 Retroviral infections

Virus production

Day 0; Plate 2.5 x 10^6 Phoenix packaging cells in 9 ml medium/10 cm dish in the afternoon. It is very important to have good single cells suspensions (trypsinize well) and to evenly distribute the cells. Day1; Transfect cells with 20 μg DNA (±24 hrs after plating), using CaPO4 precipitation. At the moment of transfection the cell density should be ± 40-50% such that the cells will be about 90% confluent at Day 3 and completely confluent at Day 4. In a 2 ml eppendorf tube mix: 50 μl of 2.5 M CaCl2, 20 μg DNA (Qiagen prep purified,

pBabe vector) and makes up to total of 500µl with serum free DMEM. While vortexing the tube, slowly add 500µl 2x HBS drop by drop. Add the 1 ml mix drop by drop to the cells in medium and evenly distribute by swirling the plate. Place cells back in incubator. Day 2; Change medium 5-20 hours after transfection. Late in the afternoon or in evening replace medium with 9 ml of fresh medium. Day3; Collect first supernatant (this is 48 hrs after transfection and not later than 24 hrs after changing medium): Remove virus-containing medium and set aside for a moment to supply the packaging cells with 9 mls of fresh medium (1-2 plates at a time). 2. Filter the virus-containing medium through a 0.45 µm filter and immediately use for infection.

Retroviral Infection

Day 0 Plate cells at a density such that they are growing well for the entire duration of infection and will be confluent at the end of infection (Day 3), about 40% confluent. For single round of infection: Day 1; If target cells grow in same medium as packaging cells: Add 4µ g/ml Polybrene to virus (from 100 x stock), remove medium and cover cells with virus + Polybrene: 12-well plate: 300-500 µl/well 6-well plate: 750-1000 µl/well T25/5 cm dish: 1.5 ml 10 cm dish: 4.5 ml. If target cells grow in different medium as packaging cells (not in DMEM): Perform the infection using viral supernatant in Phoenix cell medium that is only supplemented with polybrene and not with the medium of the target cells. This means that the infection efficiency will drop because the cells are not growing as optimal as they could be, but this method is better than using virus in other medium. Day 1; 6-8 hrs later: Add medium (the type in which the target cells grow) to the virus incubations to dilute the Polybrene which is toxic at concentrations higher than 2 µg/ml. e.g to: 500 µl of virus, add 750 µl of medium 1 ml of virus, add 1.5 ml medium 1.5 ml of virus, add 2 ml medium 4.5 ml of virus, add 6 ml medium Grow cells like this for 48 hrs after start of infection (until Day 3) before splitting or starting antibiotic selection.

Required Solutions

Polybrene
Stock is 10,000x in medium stored at 4°C (40 mg/ml) and user stock is 100x.
Final concentration is 4 µg/ml.
Medium for Phoenix cells:
DMEM, 10% FBS, 1% Non-essential amino acids, 1% Pen-Strep, 1% Glutamate Store medium at 4°C, warm to 37°C before use.
2.5 M CaCl2, Filter sterilize and store aliquots at -20°C
2x HBS
50 mM HEPES pH 7.05
10 mM KCl
12 mM Dextrose
280 mM NaCl
1.5 mM Na2HPO4 (FW 141.96)

The final pH of the solution should be 7.05 +/- 0.05. Filter through a 0.2 µm filter, aliquot, and store at -20°C. Try to avoid multiple freeze/thaw cycles. To thaw, warm to room temperature and invert or vortex the tube to achieve uniform mixing. Although it is unclear why this occurs, the ability of the 2x HBS solution to produce working CaPO4 precipitates deteriorates after 6 months to one year, even when the 2x HBS solution is stored at -20°C.

3.6 Transfection of cells using Fugine

One day prior to transfection, 2 x 10^5 both HPV38 and HNSCC cells cells were seeded into sterile 6-well plates and cultured in 2 ml of DMEM supplemented with 10% FCS, 1% L-glutamine, and 1% of penicillin and streptomycin (FAD medium was used for HPV38 keratinocyts). Following, overnight incubation at 37°C/5% CO2, with humidity, 60% confluency resulted for each well. Cells were then transfected with siRNA against p53 and scramble, using FuGENE® 6 transfection reagents. According to transfection protocols for FuGENE® 6 Transfection Reagent, DNA solution containing 10 µg siRNA was diluted in 100 µl of serum-free Opti-MEM, and various transfection reagent:DNA ratios were prepared for each transfection. The mixtures were then vortexed and incubated at room temperature for 15 minutes. In the meantime, the culture medium in the 6-well plates was removed and cells were washed with 1x PBS. 900 µl of fresh serum-free Opti-MEM and 100 µl of transfection mixture were mixed and added to each well. Fresh medium was added following overnight incubation. All cells were then cultured for another 2 days. Two days after transfection, cells were washed twice with 1x PBS and collected for protein and RNA isolation and senescence detection procedure was followed.

3.7 Immunofluorescence

To be able to detect antigens in living cells, the cells can be fixed and stained with the desired primary antibodies. The secondary antibody, which binds to the first, can be labelled with various dyes, which can in turn be detected in the microscope, when excited with light of certain wavelengths. Cells were seeded into 6-well plates, each well containing glass cover slips. To reach a confluency of approx. 60% before transfection $1x10^5$-$1.5x10^5$ cells were seeded 3 days before or $2.5x10^5$ cells were seeded the day before transfection. 24-30 h post transfection cells were carefully washed 3 times with 37°C warm PBS and fixed using 200 µl ice cold 4 % paraformaldehyde (PFA) in PBS for 20 min. For permeabilisation the fixed cells were washed and incubated exactly for 2 minutes with ice cold 0.2 % Triton X-100 in PBS. Cells were washed immediately 3 times with 1 % BSA in PBS. Blocking was done by an incubation of at least 10 min with 1 % BSA in PBS. The primary antibody (dilution as recommended) was incubated by pipetting 20-30 µl drops of antibody onto a clean surface and placing cells on cover slips facing down into

the drop. After 1 h of incubation at 37°C in a humidified atmosphere, cells were washed three times carefully with 1 % BSA in PBS. Before incubation with secondary antibody the cells were blocked for 10 min with 1 % BSA in PBS. Incubation was done in the dark to avoid bleaching of the fluorescent dyes for 45 min. Cells were washed twice with 1 % BSA in PBS and twice with PBS. Cover slips were finally embedded in approximately 15 µl Fluoromount G (Biozol, Germany) put on slide, cover slip sealed with nail varnish and slide was labelled. Samples were viewed by indirect immunofluorescence microscopy using the confocal scanning system MicroRadiance (Biorad, Great Britain) in combination with a Zeiss Axiophot microscope. The following filters were used for FITC- and TRITC-derived fluorescence: excitation for FITC at 488 nm and TRITC at 543 nm; emission for FITC at 515-530 nm and TRITC at >570 nm.

3.8 FACS analysis

Flow cytometry is a means of measuring certain physical and chemical characteristics of cells or particles as they travel in suspension one by one past a sensing point. The fluorescence activated cell sorter (FACS) consists of a light source, collection optics, electronics and a computer to translate signals to data. In most cytometers the light source of choice is a laser which emits coherent light at a specified wavelength. Scattered and emitted fluorescent light is collected by two lenses (one set in front of the light source and one set at right angles) and by a series of optics, beam splitters and filters, specific bands of fluorescence can be measured. Physical characteristics such as cell size, shape and internal complexity can be measured and any cell component or function that can be detected by a fluorescent compound can also be examined.

3.8.1 Apoptosis assay

Estimating the amount of apoptotic cells in a population can be done through measuring the DNA fragmentation. DNA fragmentation is a late characteristic of a cell undergoing apoptosis. Specific endonucleases cleave the DNA in the internucleosome linker region, producing single and multiple nucleosomes (180-200 bp). To detect the amount of DNA a dye that intercalates into the DNA, propidium iodide (PI) is used. The dye is excited with a laser and the emitted signal strength corresponds to the DNA content in each cell. The output is displayed in a histogram with the amount of counts on the Y-axis and the correspondingly emitted signal on a logarithmic scale on the X-axis. Cells that have duplicated their DNA (G2-phase) show a signal double as strong as cells in G1/G0- phase, revealing two peaks linked by a “valley” corresponding to cells increasing their DNA content (S-phase). Cells with reduced DNA content correspond to signals left of the G1/G0-peak (sub-G1). The cells were treated with different drugs for specifict time points. After treatment of drug of interest and incubation period cells were washed with ice cold PBSm the cells were

trypsinised and resuspended in fresh medium. After transferring the cell suspension into a centrifuge tube, the cells were spun for 10 min at 1,100 g, washed twice with cold PBS and finally dissolved in PI-solution. The cells were incubated in the dark for 30 min. through this treatment the plasma membrane was solubilised leaving the nucleus whole and allowing the dye to enter the nucleus. Using laser light with a 620 nm filter the DNA-intercalated propidium iodide emits a fluorescence that could be detected in the FACS (fluorescence activated cell sorter). 10,000 cells were counted for one data set. The data evaluation was done with the computer program CellQuest. PI-solution: 0.1 % Triton-X-100 0.1 % sodium citrate 50 µg/ml propidium iodide in Aqua bidest. Another method we used that, Apoptosis was evaluated using the Annexin V-FITC detection kit (BD Transduction) and by propidium iodide (PI) following the manufacturer's protocol.

3.9 Immunological detection of proteins (Western blot)

Protein lysates were prepared according to standard procedures. Briefly, target cells were lysed in a buffer containing 50 mM HEPES (pH 7.5), 1% (vol/vol) Triton X-100, 150 mM NaCl, 5 mM EGTA, 50 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride, and aprotinin at 1 µg/mL. Lysates were clarified by centrifugation at 10,000 x g for 15 minutes. Equal volumes of lysates containing comparable amounts of proteins, as estimated by a modified Bradford assay (Bio-Rad, Munich, Germany) (Bradford 1976), were boiled for 5 min in 1X Laemmli sample buffer, and resolved on an SDS 10-12% polyacrylamide gel.

To detect certain proteins transferred to a membrane, polyclonal or monoclonal antibodies specifically detecting the proteins were incubated with the membranes. The bound antibodies were detected by using secondary antibodies binding to the FC part of the primary antibody. These secondary antibodies are usually covalently bound to enzymes like horseradish peroxide (HRP) or alkaline phosphatase (AP), which in turn reacts with a given substrate allowing detection. In the case of HRP, luminol breakdown is catalysed by HRP giving off light for detection on filim. To saturate the membrane with protein before antibody incubation, the membrane was blocked by 1 h incubation in blocking buffer under agitation. The membrane was placed in a plastic foil and the first antibody, diluted in blocking buffer, was added. The foil was sealed and attached to a tumbling device, ensuring the even distribution of antibody solution over the entire membrane during the 1 hour incubation period at room temperature or over night depends upon for different primary antibody recomendation. After a 15 min wash with blocking buffer and a 15 min wash with PBS-T the blot was agitated twice in PBS-M (PBS with milk) for 5 min. The membrane was once again inserted in a foil with the secondary antibody diluted in blocking buffer and incubated during constant rotation for 1 h at RT. The membrane was washed as before, then twice in PBS. Finally, the membrane was incubated with a 1:1 mixture of enhanced luminal reagent and

oxidising reagent from the Renaissance Chemiluminescence Kit (NEN) for approximatly 2 min. Excess solution was removed and the membrane was placed in a film cassette and covered with a plastic foil. In the developing chamber a film was exposed to the membrane. Through different exposure times the intensity of the signals could be optimised. The films were developed, fixed and dried. For desitometric analysis the films were scanned and evaluated with the program Adobe Photoshop.

Blocking buffer:
1XPBS
0.05 % Tween 20
5 % (w/v) milk powder
PBS-T: PBS+0.05 % Tween 20
PBS-M: PBS+5 % (w/v) milk powder

Immunoblot analyses were carried out using the following antibodies: anti-human p53 (NCL- M1; Novocastra Laboratories Ltd, Newcastle upon Tyne, UK), anti-mouse p53 Do-1 from Santa Cruz Biotechnology (USA), anti-mouse p53 (NCL-CM5; Novocastra Laboratories), anti-phospho-p53 (Phospho-p53 Antibody Sampler kit; Cell Signaling, OZYME, Saint Quentin Yvelines, France), anti-p73 (Anti-p73 Ab-1; Calbiochem, Fontenay sous Bois Cedex, France) and (IMG-259; Imgenex, CliniSciences, Montrouge, France). Immunoprecipitation was performed using anti-p53 (NCL-CM1) or anti-p73 (Ab-1) antibody. The monoclonal antibody anti-EGFR was provided by BD Transduction Laboratories (USA); anti-ß-actin antibody was purchased from Sigma (Milan, Italy); antiphosphotyrosine PY99, anti-Fhit, anti-PTEN, anti-VEGFR2, and anti-ß-tubulin antibodies were provided by Santa Cruz Biotechnology (USA); anti-AKT, anti-phospho-AKT, anti- ERK1/2 and anti-phospho-ERK1/2 were provided by Cell Signaling (USA); anti-ABCG2 (human) MAb (BXP-21) by Alexis Corporation; and p53 polyclonal antibody NCL-CM1 and p63 polyclonal antibody were from Novocastra. A mouse and a rabbit HRP (Amersham Pharmacia Biotech, Uppsala Sweden) were used as secondary antibodies. SCO1 and SCO2 antibody as a gift from Dr. Hwang PM, National Institutes of Health, Bethesda, MD 20892, USA. TIGER mouse monoclonal antibody as a gift from Dr. Vousden KH, The Beatson Institute for Cancer Research, Switchback Road, Glasgow G61 1BD, UK.

3.9.1. Stripping of membranes for reprobing

To use a different antibody for the same blot the antibodies that bound during the first immunological detection must be removed. The proteins are bound tightly to the membrane, whereas the antibodies (primary and secondary) can be removed by use of detergent (SDS), reducing agent (β-mercaptoethanol) and heat. The signals obtainable using a reprobed membrane are weaker than for “fresh” blots, nevertheless signals are usually very good. The protocol was

adapted from (Kaufmann et al., 1987). Antibodies were stripped with Erasure buffer by incubating the blot for 30-90 min at 70°C in a water bath. The blot was washed twice in TS buffer for 10 min each. Finally, the membrane was blocked in blocking buffer for 2.5 h and was ready for primary antibody incubation.

Erasure buffer: 2 % (w/v) SDS
62.5 mM Tris-HCl pH 6.8
100 mM β-mercaptoethanol
TS buffer: 10 mM Tris-HCl pH 7.4
150 mM NaCl

3.10 Immunoprecipitation (IP)

Immunoprecipitation (IP) is a method to use a specific antibody against a protein of interest and isolate the protein-antibody complex from the rest of the cell lysate. The Fc part of the antibody binds to a *Staphylococcus* protein A or *Streptococcus* protein G, which is covalently linked to a carrier matrix like sepharose. The solid phase matrix brings the antigen-antibody complex out of solution. This precipitate can be washed, centrifuged and subsequently analysed by Western blot. This method is usually used to analyse protein-protein interactions, but can also be used to detect proteins. To obtain sufficient cell lysate for the IP 8 million adherent cells were used, which corresponds to approximatly 1 culture dish of 145 mm diameter. The cells should be roughly 90 % confluent. After removal of the cell culture medium, the cell layer was washed once in PBS. To every culture dish 4 ml ice cold PBS was added, cells were scraped off with a rubber policeman and transferred into a 50 ml tube. The cells were centrifuged at 4°C for 5 min at 1,000 g. The pellet was dissolved in 3 ml IP lysis buffer and transferred to the grinding chamber of a Potter-Elvehjem style homogeniser. Using the pestle the tissue was homogenised, transferred to a 15 ml tube and the homogeniser rinsed with IP lysis buffer. The homogenate was turned on a wheel for 20 min at 4°C. 120 µl slurry of protein G agarose (for monoclonal antibodies) or protein A agarose (for polyclonal antibodies) was equilibrated in 400 µl IP lysis buffer, later added to the lysate and incubated for 1 hour on the wheel at 4°C. This procedure of preincubating the lysate removes proteins that bind non-specifically to protein A or G agarose. Through centrifugation at 10,000 g for 10 min at 4°C the beads were sedimented, the supernatant was carefully removed and used for the IP. This should yield approx. a protein concentration of 1-5 µg/µl.
For an IP, 1 µg of antibody was added per 100 µg protein supernatant. The same amount of protein supernatant is used as negative control with an unspecific antibody (e.g. α-GST). The antibodies were incubated during constant rotation for 1 h at 4°C. The formed antibody-protein complexes were bound by addition of 30 µl Protein A agarose and could be removed by a 10

min centrifugation step at 10,000 g for 4°C. The supernatant was discarded and the pellet was washed 4 times with 1 ml IP lysis buffer. After the last washing step the pellet was resuspended in 50 µl SDS loading buffer. Probes were boiled, run on an SDS-PAGE and analysed by Western blot using an antibody directed against the protein interaction partners of the precipitated protein.

IP lysis buffer: 50 mM HEPES pH 7.0
150 mM NaCl
0.1 % NP-40
10 mM β-glycerophosphate
100 µM Na3VO4
0.2 mM PMSF
1 mM NaF
10 µg/ml Aprotinin

3.11 DNA-Sequencing

Knowledge of the exact sequence of DNA enables detection of mutations, like frame-shifts and point mutants, not detectable by restriction digests. The DNA sequences were analysed through an improved technique of the dideoxy mediated chain termination method (Sanger et al., 1977) by a commercial biotech firm. The sequencing could deliver a sequence accuracy of over 99%. The DNA used sequencing acts as a template for the enzymatic synthesis of new DNA. The sequencing primer defines the starting point of the sequencing reaction on the template DNA. In the sequencing reaction, containing DNA polymerase I, a certain fraction of the nucleotides are fluorescence-labelled dideoxynucleotides, which block further chain elongation, as they do not possess a 3´OH. The ratios of deoxy- and dideoxynucleotides are such that a finite probability is created for a dideoxynucleotide to be incorporated in place of the usual deoxynucleotide at each nucleotide position on the growing chain, resulting in a population of truncated fragments. Every dideoxynucleotide (ddATP, ddCTP, ddGTP and ddTTP) is coupled to a different dye and can hence be distinguished later. The various DNA fragments are then separated electrophoretically on a sequencing gel and analysed by exciting the dye with laser light. The emitted signals are saved as a diagram, which can be translated into the DNA sequence by a special software program.

Mutational analysis of EGFR. Genomic DNA was extracted from each cell line, using QIAamp DNA Blood Midi Kit (Qiagen) following the manufacturer's instructions. DNA was quantified and mutational analysis was performed from EGFR exons 18 to 21 and in exons 27 and 28. EGFR coding exon was amplified by PCR using the primers described in Table I. PCR amplification was carried out in a total volume of 50 µl containing 150 ng of genomic DNA as template, 1X reaction buffer, 1.5-3 mM magnesium chloride, 200 mM dNTP, 20-50 pmol of each PCR primer and 0.25 U AmpliTaq Gold.

After PCR amplifications, 5 µl of the reaction product was analysed via gel electrophoresis and ethidium bromide staining. DNA sequencing was performed on both strands of two independent PCR products by cycle sequencing on an ABI PRISM 310 automated cycle sequencer, using the Terminator Cycle Sequencing Kit according to the manufacturer's protocol (Applied Biosystems, CA). Whenever an alteration was identified, a new DNA aliquot from the same cell line was sequenced to confirm the result. The variants found in the sequence were characterized and compared to those in the online databases, EntrezSNP (http://www.ncbi.nlm.nih.gov) and Ensembl (www.ensembl.org).

3.12 Reverse-Transcription PCR

Each cell line was collected, the RNA was extracted with Absolutely RNA miniprep kit (Stratagene) and Five micrograms of RNA were converted to cDNA using the First strand cDNA synthesis kit (Fermentas MBI). The primers used for the PCR were as follows: ΔNp73, 5'-ACC ATG CTG TAC GTC GGT GAC CCC-3' (forward) and 5'-GCG ACA TGG TGT CGA AGG TGG AGC-3' (reverse); ΔNp63, 5'-TGC CCA GAC TCA ATT TAG TGA G-3' (forward) and 5'-AGA GAG AGC ATC GAA GGT GGA G-3' (reverse); TAp63, 5'-GAC CTG AGT GAC CCC ATG TG-3' (forward) and 5'-CGG GTG ATG GAG AGA GAG CA-3' (reverse). As a control, the following human GAP-DH was used: 5'-AAG GTG GTG AAG CAG GCG T-3' and 5'-GAG GAG TGG GTG TCG CTG TT-3'. Annealing temperatures (Ta) and number of cycles are specific for each isoform: ΔNp63, 25 cycles and Ta 64°C; TAp63, 35 cycles and Ta 58°C; ΔNp73, 40 cycles Ta at 62°C; TAp73, 35 cycles and Ta at 61°C and rest of the PCR amplification consisted of (94°C for 30 seconds, Ta for 30 seconds, and 72°C for 30 seconds) followed by incubation at 72°C for 7 minutes. The bands were visualized by ethidium bromide staining.

3.13 Determinations of human papilloma virus (HPV) infection by reverse line blot hybridization.

The presence of human papilloma virus HPV (type 4, 5, 8, 9, 12, 14, 15, 17,19, 20, 21, 22, 23, 24, 26, 36, 37, 38, 47, 48, 49, 50, 60 and 65) DNA was determined by reverse line blot hybridization previously described by Kleter et al. (Kleter et al., 1999; Melchers et al., 1999; Quint et al., 2001) Oligonucleotide probes specific for 24 different Human Papillomavirus types are covalently attached to a membrane in parallel lines using a miniblotter. The templates to analyze are DNA extracts previously labeled with Biotin using PCR. The labeled-PCR products are then loaded on the membrane in slots perpendicular to the oligo lines in order to allow all the samples to interact with the HPV probes. Hybridization takes place in the miniblotter and is visualized using a peroxidase labeled streptavidine, which interacts with the biotin of the PCR products, followed by chemiluminescence's detection.

3.14 Statistical Analysis

All the experiments were performed in triplicates and the average results of three independent assays ± standard deviation are indicated. Student's *t* test was used to assess if null hypothesis is true. All *P* values were two-sided, and differences were statistically significant at $P <. 02$. To evaluate the effect of a drugs induced IC_{50} values, CalcuSyn (Version 1.1.1 1996, Biosoft, Cambridge, UK) was used, a program based on the method of Chou and Talalay (Chou and Talalay, 1984).

4. RESULTS AND DISCUSSION

4.1 In-vitro Model of Head and Neck Squamous Cell Carcinoma (HNSCC); Twelve Novel Cell Lines (manuscript C)

Establishment of cancer cell lines is very important in cancer research to understand several molecular pathways and perform different drug screening analyzis which cannot be performed invivo for any cancers including head and neck cancer (Lin et al. 2007). As an in-vitro model of Head and Neck Squamous Cell Carcinoma (HNSCC), we have selected a large panel of cell lines derived from different anatomical regions (Figure 9 and 10) of Head and Neck Cancer. Twelve cell lines consisted of larynx (one case, HNC-150), oral cavity (six cases, HNC-91, -97, -124, -160, -199, -212), tonsil (three cases, HNC-41, -206, -211), hypopharynx (one case, HNC- 180) and paranasal sinus (one case, HNC-136) (dramatic representation in Figure 9). HNC-136 is sometimes not really considered as Head and Neck cancer cell line due to its origin from paranasal sinus.

Figure 9

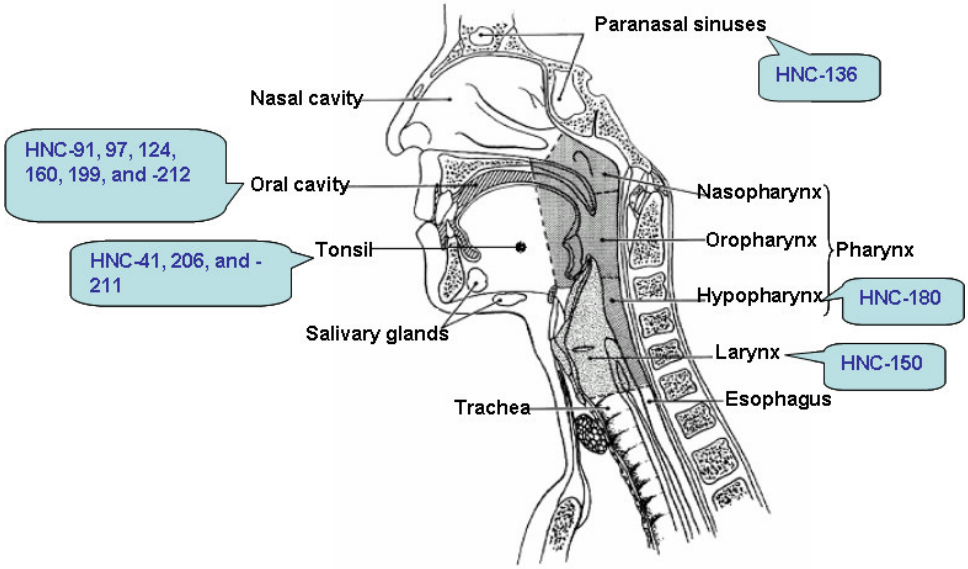


Figure 9. Dramatic representation of Human Head and Neck Anatomy. Head and Neck cancer is the term given to a variety of malignant tumors that develop in different regions of Head and Neck area as shown in figure. In this study twelve novel head and neck squamous carcinoma (HNSCC) cell lines were selected from different regions of head and neck cancers and they highlited in the figure.

Our cell lines were undergone several passage numbers as indicated in Figure 10 before utilization in experimental analyisis.

Figure 10

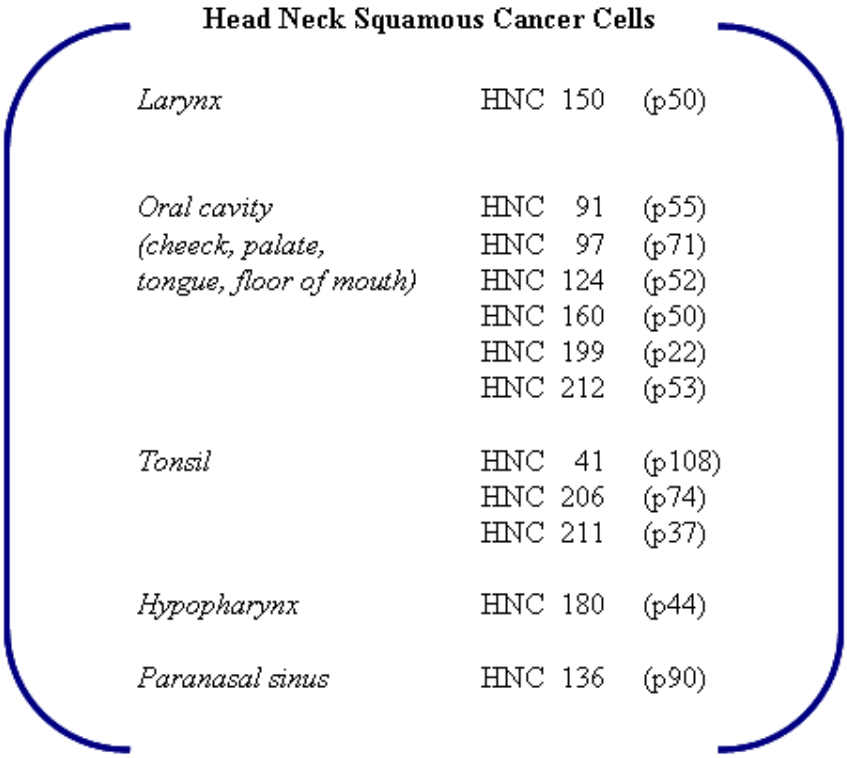


Figure. 10. Different Head and Neck tissues, from which cell lines were derived and their minimum passage numbers are undergone before utilization of different experimental procedures are drawn.

These cell lines are now available for a variety of studies designed to broaden our understanding of the biology of the head and neck cancer. Survival rates for patients with cancers of the head and neck have not been improving recently, and there is a need for new and innovative therapeutic approaches in addition to surgery, radiation, and chemotherapy. These can only be developed on the basis of an improved knowledge of the tumor properties and its behaviour in vivo and *in vitro.* Clearly, well-characterized cell lines are a valuable resource for such studies.

4.2 General Characterization of Head and Neck Cancer Cell Lines

4.2.1 Phenotypic features of head and neck cancer cell lines

Most cell lines have shown their own phenotypic characteristics and growth properties, for example HNC-136 cells are round-shaped ones grown in a clustered way but HNC-180, long-shaped cells with a homogeneous distribution (Figure. 11).

Figure 11

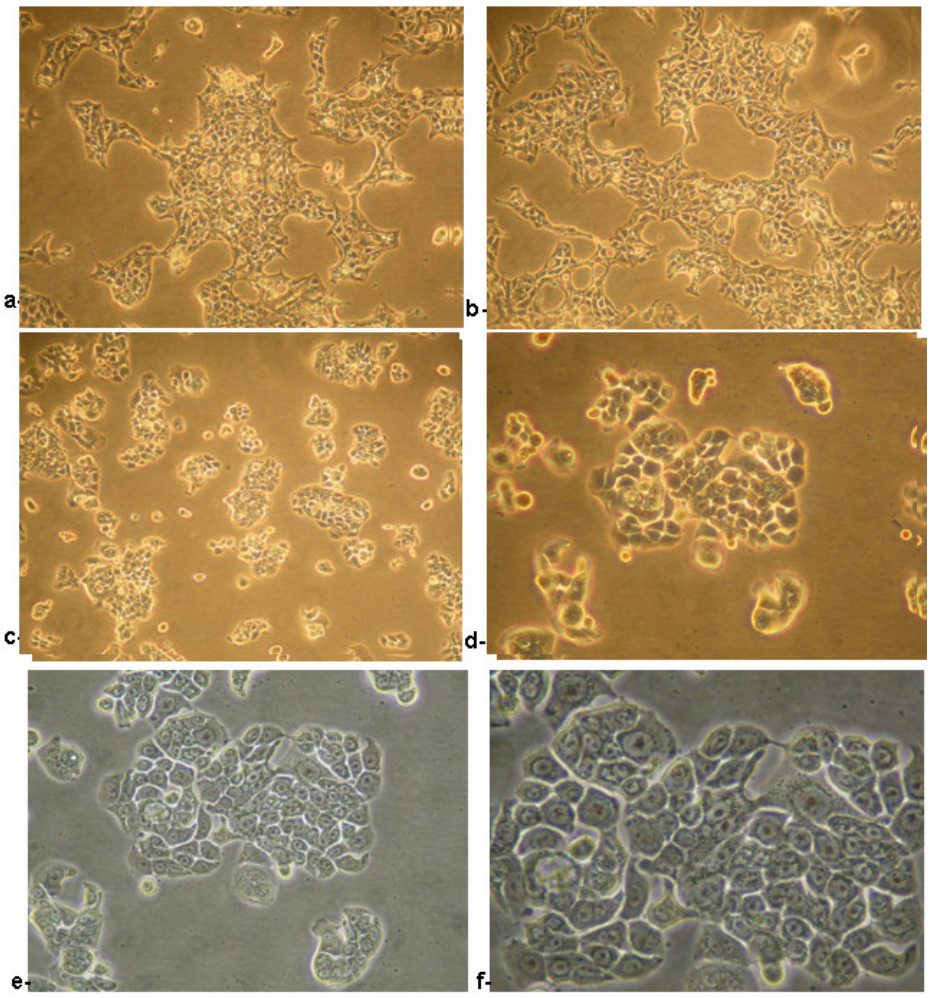


Fig.11. a-, b- Cell line 180, different magnifications. c-, d-, e-, f- Cell line 136, different magnifications. Cell lines derive from different tissues in the head and neck region (larynx, hypopharinyx, paranasal sinus, oral cavity, etc.) and this is the reason why the phenotypes of the cells are extremely various. In the two examples above, it is possible to see long-shaped cells with a homogeneous distribution (fig. a-, b-) and round-shaped ones grown in a clustered way (fig. c-, d-, e-, f-)

This diffrence in cell phenotype is maily due to its origin deriving from different anatomical regions of head and neck cancer. Figure 11 shows cell lines HNC-180 and HNC-136 derived from hypopharinyx and paranasal sinus, respectively.

4.2.2 Doubling time population and DNA ploidy analysis

Another general characteristic was that all cell lines grew as monolayer but they have shown significant difference in doubling time population, ranging from 18 to 72 hours (Table 3). For example, HNC-212 was very slow in growth and its doubling time was around more than 72 hours. In contrast, HNC-136 was very fast growing cell line and its doubling time was around 18-24 hours. Since all cell lines were established with the same culture conditions, these data generally indicate that different cellular pathways are altered in different cell lines. Cell cycle analysis reported in table 3 shows that each cell line has peculiar distribution in the G0/G1-, S- and G2/M-phase and, as respect to lymphocytes all of them are aneuploid with a DNA index ranging in-between 1.13 and 1.75.

Table 3

Cell Lines	DNA Index	Cell Cycle Analysis (% of cell at different phase of cell cycle)			Ploidy	Doubling Time in Hours
		G0/G1	S	G2/M		
HNC-41	1.54	65.45	22.05	17.50	Aneuploid	18-24
HNC-91	1.76	73.48	6.63	19.89	Aneuploid	24-48
HNC-97	1.48	73.95	4.81	21.24	Aneuploid	24-48
HNC-124	1.41	54.95	19.77	25.28	Aneuploid	>48
HNC-136	1.48	63.01	6.93	30.06	Aneuploid	18-24
HNC-150	1.43	74.60	5.99	19.41	Aneuploid	24-48
HNC-160	1.13	66.62	13.81	19.56	Aneuploid	24-48
HNC-180	1.75	47.64	33.63	18.73	Aneuploid	>48
HNC-199	1.37	60.70	16.21	23.08	Aneuploid	18-24
HNC-206	1.36	51.77	30.07	18.17	Aneuploid	24-48
HNC-211	1.58	63.63	19.26	17.08	Aneuploid	24-48
HNC-212	1.67	60.65	4.06	16.29	Aneuploid	>72

Table. 3. General Cell cycle analyses and DNA index of each cell lines were determined by FACS analysis. Exponentially growing each cell lines were collected and stained with propedium iodide and DNA index was determined with respect to normal lymphocytes. Each cell lines doubling time population was determined by direct cell counting.

Aneuploidy, an abnormal nuclear DNA content, is considered almost positive evidence of any malignancy including HNSCC (Rubio Bueno et al. 1998). As our all cell lines analyzed were aneuploid indicating that during tumour

progression there must be an acquisition and accumulation of chromosomal aberrations in Head and Neck Cancer.

4.3 HPV Detection

High-risk human papillomaviruses (HPVs) have been proposed to be associated with a subset of head and neck cancers (HNSCCs). All cell lines were previously tested for the presence of these HPV types. No DNA of the high-risk HPV was detected in all cell lines. Recent epidemiologic studies have reported associations between markers of ß-HPV infection (presence of viral antibodies in serum and presence of viral DNA in plucked eyebrow hairs) and actinic keratosis (AK) and Cutaneous squamous cell carcinoma (SCC) independently (de Villiers et al. 1997). Therefore, we examined whether HNSCC cell lines were positive for cutaneaus HPV types DNA (type 4, 5, 8, 9, 12, 14, 15, 17,19, 20, 21, 22, 23, 24, 26, 36, 37, 38, 47, 48, 49, 50, 60 and 65) by reverse line blot hybridization (Figure 12). In order to check for HPV presence, reverse line blot hybridization was performed on DNA extracts from the twelve cancer cell lines. The membrane was already prepared with HPV probes loaded vertically; DNA extracts from the twelve cell lines were loaded horizontally on the membrane in order to cross-interact with the HPV DNA probe (Figure 12). Our analysis revealed that all cell lines used for this study were negative for most prevalent mucosal and cutaneous HPV types.

Figure 12

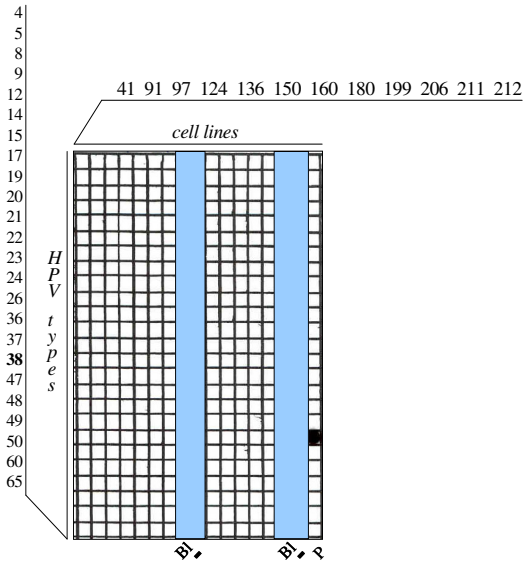


Figure 12. HPV typing. Dramatic representation of reverse line blots hybridization for HPV typing. The blotting didn't give any positive results indicating that there is no evidence of HPV infection in the cell lines under study. On the membrane the DNA extracts from the 12 cell lines have been loaded horizontally in order to cross with the specific HPV probes loaded vertically. The light blue lanes are the blanks and the negative controls, while the HPV38 was used as positive control (the black spot).

HPV negativity in our HNSCC cell lines analysed for prevalent mucosal and cutaneus HPVs inderectly suggest that HPV infection is an early event in HNSCC development and virus DNA integration may not be strictly necessary for HNSCC progression when compared with cervical cancer, where viral DNA integration to host genome is most prevalent.

Part I

4.4 Characterization of "p53 family net work" in twelve novel HNSCC cell lines

We analyzed the expression profiles of p53 family members in our squamous cell carcinomas of the head and neck (HNSCC) derived cell lines. Each cell line was collected, the RNA was extracted with Absolutely RNA miniprep kit (Stratagene) and reverse transcription was carried out using the First strand cDNA synthesis kit (Fermentas MBI). In order to evaluate the expression levels of the p53 homologous and isoforms, polymerase chain reactions (PCR) were performed using isoform-specific primers. In particular the levels of TAp63, ΔNp63, TAp73, ΔNp73 were analysed. A PCR for the housekeeping gene GAPDH was performed on all the samples in order to normalize the quantity of the cDNA. For comparing our RT data with protein levels, western blot analysis were performed for all p53 homologous and isoforms. In the majority of HNSCC cells different p63/p73 isoforms were expressed with cell-line-specific patterns for composition and intensity of transcript expression and these results are descriped separately in the following sections.

4.4.1 TAp63 expression and over-expression of ΔNp63 in HNSCC cell lines

The human p63 gene encodes a series of proteins that differ in their N- and/or C-terminal sequences and have widely differing properties in promoting or repressing p53-related functions such as growth arrest and apoptosis. In addition, p63 has important roles in the maintenance and differentiation of epithelial cell populations. In our study noticeably ΔNp63 was found over-expressed already in the first PCR in almost all the cell lines (Figure 13-a). After the NESTED PCR two other cell lines appeared to be positive except HNC-91 and HNC-136 (Figure 13-b).

The expression levels of TAp63 were also analysed and it was found that HNC-41 and HNC-206 highly expressed Tap63 at RNA level (Figure 14) and its increased mRNA expression correlated with protein in HNC-41 but HNC-206 has shown comparatively less amount of protein. (Figure 15).

Figure 13

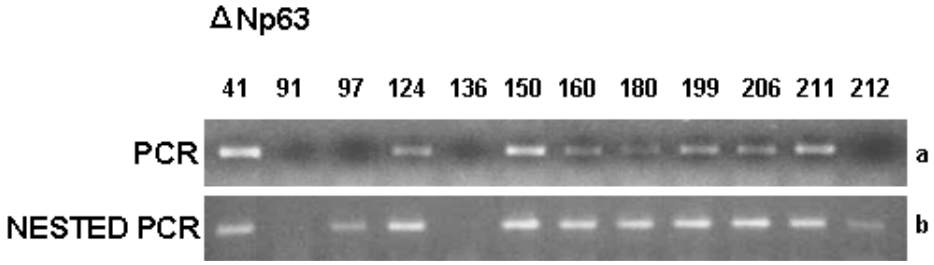


Figure 13. PCR on ΔNp63. The first PCR gave several positive results, which have been confirmed in the NESTED PCR with the addition of two other positive bands in the cell lines 97 and 212.

Figure 14

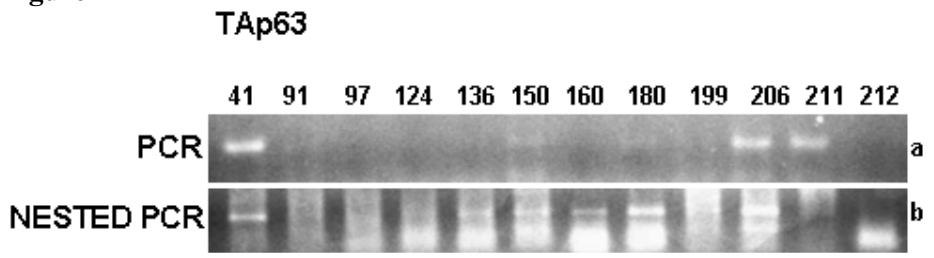


Figure 14. PCR on TAp63. Even in this case the increase in the number of positives and the enhancement of the intensity of the signals is evident between the first PCR and the NESTED. Only in the 211-cell line there is a regression of positivity, which may be an artefact due to the excess of template for that specific sample.

4.4.1.1 TAp63 and ΔNp63 protein analysis by immunoblotting

Protein extract were prepared from each cell line and analyzed by immunoblotting with a polyclonal antibody against p63 (Figure 15). Our immunoblot results confirmed that TAp63 and ΔNp63 are not expressed in HNC-91 and HNC-136 at RNA and protein levels.

Figure 15

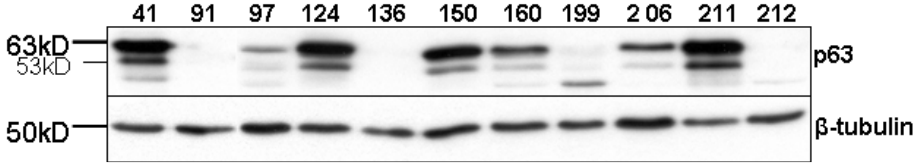


Figure 15. Westernblot for p63. 100 μg of each protein extract were loaded on a 12% polyacrilamide gel, transfered to a PVDF membrane and immunostained overnight by a polyclonal antibody against p63 used at the dilution 1:1000. An immunostaining for β-tubulin was performed on the same membrane as a loading control.

Our invitro results supports the previous finding that Squamous cell carcinomas of the head and neck (SCCHN), express high levels of ΔNp63 isoform compared to normal tissue from the same patients, suggesting a role for these isoforms in the pathogenesis of this common human malignancy.

4.4.2 p53 homologue Tap73 and its isoform ΔNp73 expression in HNSCC cell lines

High levels of ΔNp73 mRNA were found expressed in only two cell lines, the 150 and the 211 (Figure 16-a). In order to amplify the signal, a NESTED PCR was performed on the products from the previous PCR and the enhancement was significant since the 75% of all the cell lines appeared to be positive for ΔNp73 (Figure 16-b). The first PCR on TAp73 detected only two positive cell lines as well, the HNC 41 and the 136 (Figure 17-a). Also in this case we performed NESTED PCR, but the analylsis did not show p73 expression in additional cell lines (Figure 17-b).

Figure 16

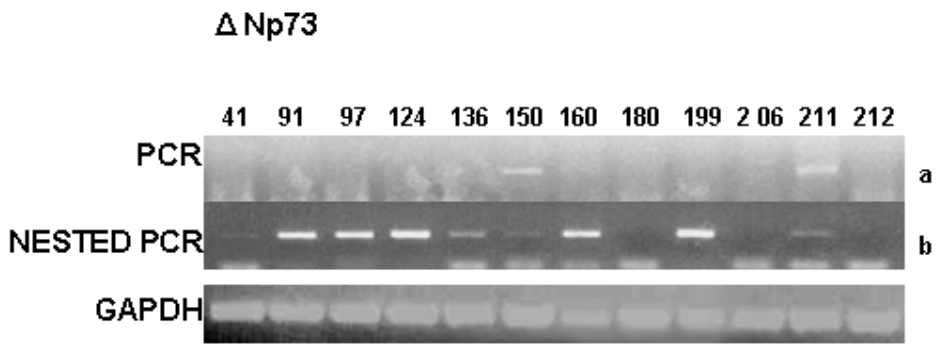


Figure 16. PCR on ΔNp73. The first PCR gave 2 positive bands, while in the NESTED PCR the outcome of positive signals is significantly enhanced. In total, 9 cell lines out of 12 appear to express ΔNp73.

Figure 17

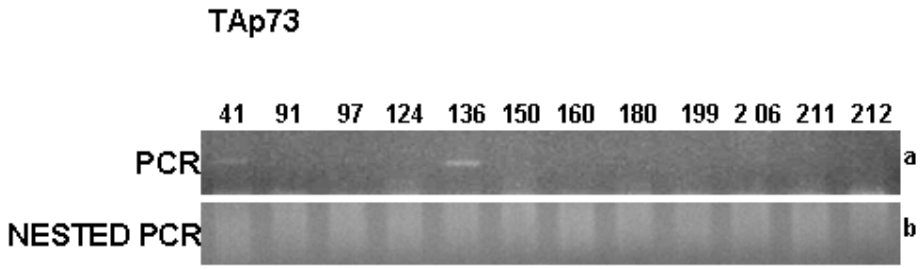


Figure 17. PCR on TAp73. In the first PCR is possible to see two positive bands, a stronger one on the 136 cell line and a weaker one on the 41 cell line. The NESTED PCR didn't give any results despite the several tests performed in different conditions.

4.4.2.1 TAp73 and ΔNp73 protein analysis by immunoblotting

To correlate with our RT-PCR data we have preformed western blotting for p73 and it revealed that HNC-206, -180 and HNC-41 accumulated both Tap73 and ΔNp73 while others were positive for only ΔNp73 (Figure 18). We couldn't perfom protein analysis of p73 in HNC-91 and 160 cell lines.

Figure 18

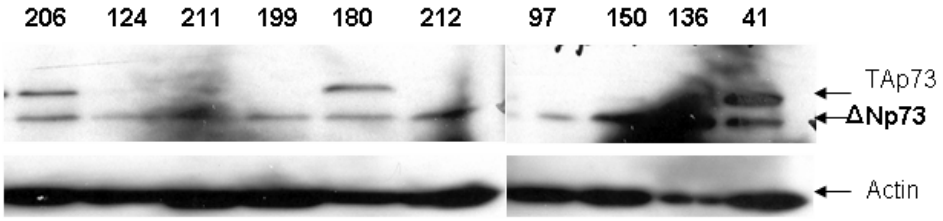


Figure 18. Western Blot analysis shows Tap73 and ΔNp73 expression in Head and Neck Cancer Cell lines.

The results from the RT-PCR and the western blots of p63 and p73 are compared in the table 4

Table 4

Cell Lines	p63 RT- PCR		p63 Western		p73 RT-PCR		p73 Western	
	ΔN	TA	ΔN	TA	ΔN	TA	ΔN	TA
41	+++	+	+++	+++	+	+	++	+++
91	-	-	-	-	++	-	Nd	Nd
97	+	-	+	+	++	-	+	-
124	+++	-	+++	+++	+++	-	++	-
136	-	+	-	-	++	++	+++	-
150	+++	+	++	+++	++	-	++	-
160	+++	+	+	++	++	-	Nd	Nd
180	+++	++	Nd	Nd	-	-	+	++
199	+++	-	-	-	++	-	+	-
206	+++	++	+	++	-	-	+	++
211	+++	+	+++	+++	++	-	++	-
212	+	-	-	-	-	-	++	-

Table 4. Results from the western blot and RT-PCR for ΔNp63, ΔNp73, TAp63 and Tap73 are compared

We have found strong expression of p73 and p63 short-variants in 75% and 83% respectively of all cell lines in contrast with the very low expression of the related full-length isoforms. This result is consistent with other data on head and neck and related tumors, where it is also very often showed strong expression of ΔN-variants of p53 homologous. In according with these results, ΔNp73 and ΔNp63 overexpression has been found in several different tissue-related cancers but not in its normal counterpart.

High levels of expression of these ΔN-variants of p53 isoforms in most of the twelve cancer cell lines suggest the possible role of these isoforms in tumorigenesis, for example through inhibition of wild type p53 functions. Surprisingly the major finding of this study was the detection of the higher expression of ΔNp63, which shows a similar pattern of expression to the one of ΔNp73. These results could suggest a more important role of ΔNp63 in tumorigenesis than what assumed in the past.

4.5 p53 status and protein levels in head and neck cancer cell lines

4.5.1 p53 mutation predispose to HNSCC

Since in more than 50% of tumors p53 is mutated, next we checked p53 status in our head and neck cancer cell lines under study. Furthermore, HNSCC with mutated TP53, may have a higher proliferative potential caused by a lack of control in G1 checkpoint. We collected the cells and performed DNA extraction. Exon 4-10 were amplified by PCR using specific primers annealing in the introns. The DNA was sequenced and screened for p53 mutations. The mutations and their location in the gene are listed in Table 5.

Table 5

Cell Lines	EXON	CODON BASE	AA CHANGE
41	WT (4-9)		
91	WT (4-9)		
97	Exon 5 Exon 7	180 GAG-AAG 248 CGG-TGG	Glu-Lys Arg-Trp
124	WT (4-9)		
136	WT (4-9)		
150	Exon 5	135 TGC-TAC	Cys-Tyr
160	Exon 6	213 CGA-CGG	Arg-Arg
180	Exon 6	220 TAT-TGT	Tyr-Cys
199	Exon 5	135 TGC-TTC	Cys-Phe
206	Exon 5	134 TTT-TCT	Phe-Ser
211	Exon 6	177-183 Del 5 codons	In frame
212	Exon 6 Exon 9	196 CGA-CCA 331 CAG-.AG	Arg-Pro Frameshift

Table 5. p53 status: mutations and wild types.

Sequence analysis revealed that 8 cells lines out of 12 harbour a mutated state of p53. Our results also support the common fact that p53 mutations are more common to DNA binding domains in HNSCC (Poeta et al. 2007). For any wild type and/or mutated genes to induce its positive or negative function it has to express at protein level and this is more stringent to p53 protein because its protein level and cellular localization determine its negative or positive roles in human cells especially the fate of immortalized cancer cells. Therefore, we have decided to determine p53 protein level in our all cell lines and their cellular localization in some selected cell lines.

4.5.2 p53 protein levels in head and neck cancer cell lines

The expression of p53 protein has been reported to be in the range of 35% to 67% in head and neck squamous cell carcinoma (HNSCC). Mutation of the p53 gene often results in over-expression of p53 protein. However, it is possible that p53 protein accumulation may be a result of alternative mechanisms leading to p53 protein stabilization. For understanding p53 protein accumulation and localization in our cell lines we have checked p53 levels by western blot analysis and to localize p53 protein we performed immunoflurescence in some selected cell lines. Our westernblot analysis showed that seven cell lines out of twelve exhibited detectable levels of p53 (HNC 97, 124, 136, 150, 199, 211, 212) and they included both mutated and wild type p53 (Figure 19). The results from the western blot analysis are listed in table 6.

Figure 19

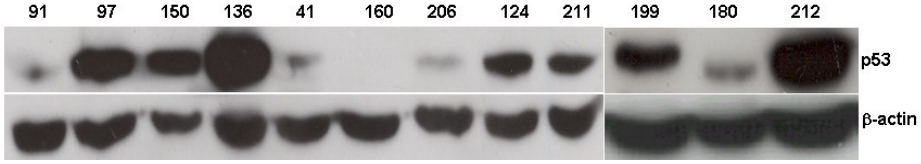


Figure19. Westernblott for p53. 50 µg of each protein extract were loaded on a 12% polyacrilamide gel, transfered to a PVDF membrane and immunostained overnight by a monoclonal antibody (DO-1) against p53 used at the dilution 1:1000. An immunostaining for β-actin was performed on the same membrane as a loading control.

Table 6

Cell Lines	p53 Levels	p53 Status
41	-	WT
91	-	WT
97	+	Mutant
124	++	WT
136	+++	WT
150	+	Mutant
160	-	Mutant
180	+	Mutant
199	+	Mutant
206	+	Mutant
211	+	Mutant
212	++	Mutant

Table 6. p53 protein expression levels in different HNSCC cell lines, WT: Wild type

p53 protein accumulation in some HNSCC cells line was interesting. Because of transdominant inhibiting isoforms, ΔNp73 and ΔNp63 would be then the truly important gene products, overexpressed and inhibiting the tumor suppressive function of p53 and its phenotypically neutral isoforms, TAp73 and TAp63. These inhibitors would antagonize the apoptotic function of p53 and of its own p53-like isoforms, either by direct competitive binding to the same cognate p53 DNA binding sites or by engaging in heterocomplexes that are defective for specific DNA binding. Therefore, it was reasonable to find high levels of ΔNp73 particularly in those cell lines that exhibit wild type p53. Of nine cell lines with a noticeable ΔNp73 expression, only three also showed expression of p53 (HNC 124, 136, 211) and only two of them harbored a wild type p53 (HNC 124, 136), whereas all the remaining have no high levels of p53 expression. We didn't find any correlation of ΔNp63 and p53 level in these cell lines because two cell lines which didn't express ΔNp63 are HNC-91 and 136, without and with accumulation wild type p53, respectevily.

4.5.3 Analysis of cellular localization of p53 protein

Cellular localization of p53 protein determines its functional activity and also to correlate with our immunoblot results, we performed immunoflorescence for p53 in some of our selected HNSCC cell lines expressing wild and mutant p53 protein. Our immunofluroscence results of HNC-41, HNC-97, HNC-136 and HNC-206 are shown in Figure 20.

Figure 20

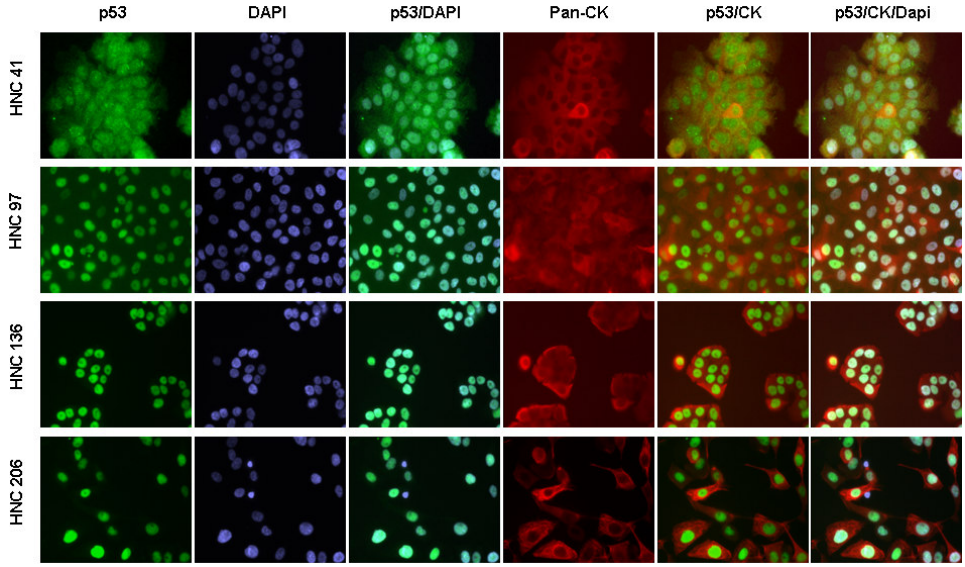


Figure 20. Immunofluroscence staining for p53. HNC-41, 97, 136, and –206 cell lines were monolayer-cultured, formaldehyde-fixed and stained with antibodies against p53 and/or Cytokeratin with or without DAPI. It is more evident that HNC136 cell line is more predominantly accumulating wild type p53 in nucleus compared with other cell lines.

Our analysis revealed that HNC-136 is highly positive for p53 protein expression and localized in the nucleus. In regards with another cell line, which harbour wild type p53 but not detected by western-blot analysis was HNC-41. Immunofluroscence staining for p53 showed slight expression but diffused throughout the cell. The observation that p53 is frequently detected in the cell nucleus suggests that nuclear localization of this protein is most likely important for p53 activity. Our results evidence that both p53 wild type and mutant cells accumulate p53 protein predominantly present in the nucleus. Negative role of mutant p53 protein in cancer is well documented; that it can co-operate with other oncogenes like ras to assist cancer cell survival and proliferation. This may be the role of nuclear p53 in HNC-97. But what the functions of wild p53 protein accumulated in the nucleus are not known in the case of HNC-136. Therefore, we have expanded our study to understand accumulated wild type p53 properties and functions in Head and Neck Cancer cells by using HPV38 mediated immortalized human keratinocytes as a prototype model.

4.6 Characterization of novel mechanisms of alteration of wild-type p53 tumor supressor function in HPV positive and negative cancer cells

p53 was initially identified as a protein associated with viral oncoproteins and later its involvement in several cell fate determining pathways was most found.

And in notion to this evident support that more than 50% of human tumors contain a mutated or deleted p53 gene. Accordingly it has also been noted that in absence of p53 mutation, viral mediated tumorogenesis, viral oncoproteins reported to inactivate p53 tumor suppressor function. High Risk Human Papilloma Virus (HPV) HPV-16 and 18 have been reported as main etiological factors, almost 90% of cervical cancers and 20 to 30% of Head and Neck cancers world-wide. Moreover, High-risk HPV early oncoprotiens E6 and E7 reported to immortalize primary human keratinocytes in-vitro. HPV 16 E6 mediated immortalization process, E6 protein reported to degrade wild type p53 by E6AP mediated ubiquitination process while E7 protein slightly accumulates p53 (Figure 21) and an unknown pathway compromises its tumor suppressor activity. Recently, it has been reported from our lab that beta type human papillomavirus (HPV38) E6/E7 mediated immortalization process leads to accumulation of wild type p53 (Figure 21). However, what is the significance of this accumulated wild type p53 in HPV38 E6 and E7 (hereafter referred to as HPV38-keratinocytes) keratinocytes is going to be revealed. One mechanism could be the selective activation of the transcription of ΔNp73, an isoform of the p53-related protein p73, which in turn inhibits the capacity of p53 to induce the transcription of genes involved in growth suppression and apoptosis (Figure 21) (Accardi et al. 2006). Moreover, its more important to understand existance of this molecular mechanism in other HPV negative cancer cell lines. Our Head and Neck Cancer cell lines characterization revealed four cell lines harboring wild type p53 and two of them were accumulating wild type p53 and ΔNp73 proteins (HNC-124 and HNC-136) (Figure 21).

Figure 21

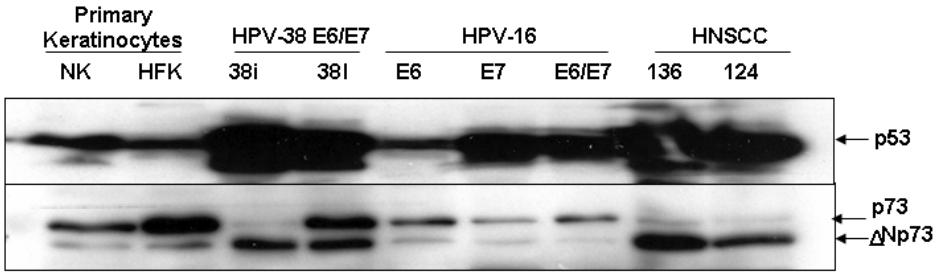


Figure 21. P53 protein levels in HPV positive and negative cancer cell lines. Human freskin keratinocytes (HFKs) expressing HPV38 E6 and E7 accumulates high levels of p53 protein (38i and 38I). HPV 16 E6 or E7 alone and E6/7 together expressing keratinocytes are also shown. Head and Neck cancer cell lines established from head and neck squamous carcinoma which are devoid of any HPV DNA (HNC-136 and-124) also showed accumulation of wild type p53.

In this regards it's very important to understand how these cancer cell lines are continuously proliferating without showing any sign of apoptosis or senescence in presence of huge amount of wild type p53 protein. Therefore, we have

focused on molecularly characterize the properties (different post translational modifications of accumulated wild type p53) and functions (down-stream events) of accumulated wild type p53 in HPV positive (HPV38) and negative cancer (HNSCC) cells.

4.6.1 Molecular properties of accumulated wild-type p53 in HPV38 and head and neck cancer cells

The well-studied post-translational modification of a single protein to date is human p53 protein. It includes p53 phosphorylations at different amino acid residues and p53 C-terminal lysines are modification by ubiquitylation, acetylation, sumoylation, neddylation and methylation. As, p53 each post-translational modification can influence its stability and functional properties, we have tried to over look on feasible post-translational modifications involved in accumulation of wild type p53 in HPV38 and HNSCC cells.

4.6.1.1 p53 ubiquitination

MDM2 is an E3 ubiquitin ligase that targets p53 for proteasomal degradation. We have tried to analyze wild type p53 accumulation in our cancer cell lines due to altered p53 ubiquitination and deregulation by MDM2. MDM2 ubiquitinates p53 prior to proteasome degradation. For understanding MDM2 and p53 cross talk in our cancer cell lines, we have analyzed basic ubiquitinated p53 status in our cell lines and its interaction with MDM2. Basic p53 ubiquitination analysis showed that according to p53 protein levels, it ubiquitinated and degraded because normal keratinocytes express low-levels of p53 is less ubiuquitinated compared with 38i and HNC-136 cells have more p53 and more ubiquitination (Figure 22). Our analysis indicates that there are basal level of p53 ubiquitination and degradation in all cell lines with respect to p53 protein levels (Figure 22).

Figure 22

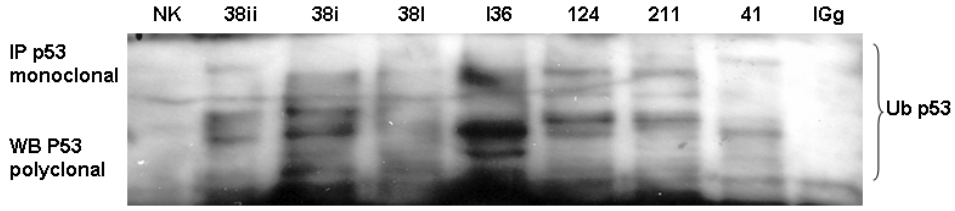


Figure 22. Basal ubiquitinated p53 status in HPV38 E6/E7 immortalized keratinocytes and selected head and neck cancer cell lines.

Next, we have analysed that these consequences are due to the altered interaction of p53 and MDM2. For understanding functional interaction between p53 and MDM2 we have immunopricipitated p53 and performed western blot for MDM2. The results revield that p53 accumulated cell lines are

less interacting with MDM2 in comparison with Normal Keratinocytes and HNC-41 cell lines which have less amount of wild type p53 protein (Figure 23).

Figure 23

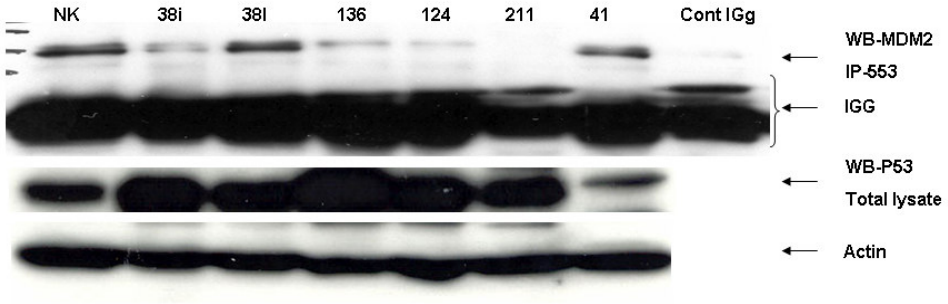


Figure 23. p53 accumulated HPV38 immortalized keratinocytes (38i and 38I) and Head and Neck cancer cell lines (HNC-136 and 124) were less interacting with MDM2.

For understanding in detail whether MDM2 protein level may be down regulated in those p53 accumulated HPV positive and negative cell lines, we have performed immunoblot for MDM2 in total cell lysate and revealed that MDM2 protein expression is less in those cells which are accumulating wild type p53 in comparison with cells (normal keratinocytes and HNC-41) containing less wild type p53 protein (data not shown). It indicates that p53 feedback loop is altered in these cell lines. Meantime, p14ARF1 has been reported to prohibit MDM2 mediated ubiquitination of p53, therefore we have analyzed p14ARF1 in the cells of our study.

4.6.1.1.1 p14ARF1 protein accumulated in HPV38 and HNC-136

Oncogenic signalling activates p53 though ARF, which, in turn, interacts with MDM2 inhibiting its p53-ubiquitin ligase activity. In this manner, ARF-dependent stabilization of p53 results in a dramatic increase in p53 activity. In our cell lines both HPV38 keratinocytes and HNC-136 are accumulating huge amount of wild type p53 and p14ARF too (Figure 24).

Figure 24

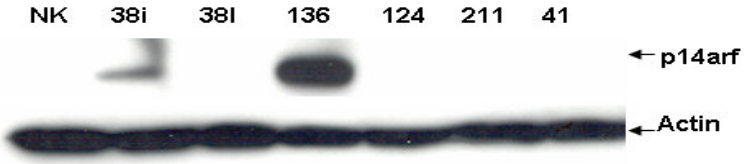


Figure 24. Western blot analysis shows p14ARF protein accumulated in HPV38 keratinocytes and HNC-136, which are accumulating huge amount of wild type p53 protein.

In our cells under study (HPV38keratinocytes and HNC-136), MDM2 protein level was very less and it's makes less significance of p14ARF interaction with MDM2 leads to p53 accumulation. It idicates that accumulated p14ARF has some assisting role in cell survival in co-operation with accumulated wild type p53 protein.

4.6.1.2 p53 sumoylation

The tumour suppressor p53 has been shown to be modified at its C-terminus with the ubiquitin-like proteins SUMO1 and NEDD8. Recently, p53 sumoylation has been proposed to induce senescence in normal human fibroblasts but apoptosis in RB (retinoblastoma)-deficient cells. Moreover, p53 sumoylation reported to influence in p53 mediated transcription activity. For understanding p53 sumoylation role in p53 protein accumulation and its functional impairement in p53 mediated tumor suppressor functions, we have analyzed sumoylated p53 status in our immortalized cell lines. Figure 31 shows that accumulated wild type p53 in HPV38 and Head and Neck cancer cells have no difference in sumoylation pattern in comparison with normal keratinocytes because none of them showed significantly sumoylated p53 (Figure 25).

Figure 25

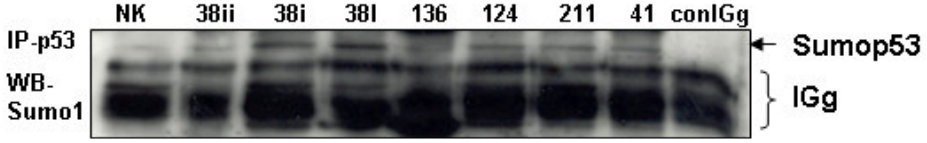


Figure 25. Basal sumoylated p53 in HPV38 immortalized keratinocytes (38ii, 38i and 38I) and other head and neck cancer cell lines. Total cell lysated from each cell lines were immunopricipitated with p53 monoclonal antibody and immunobloted with anti-sumo-1 antibody.

In addition, for understanding UV treatment enhance p53 sumoylation, we have treated our selected HPV positive and negative cancer cells for UV-B treatment. Our result shows there wasn't a significant role of p53 sumoylation leading to protein accumulation because in comparison with normal keratinocytes and p53 mutated HNC-211, wild type p53 accumulated cells didn't show any difference in sumoylation pattern with or without UVB treatment (Figure 26).

Figure 26

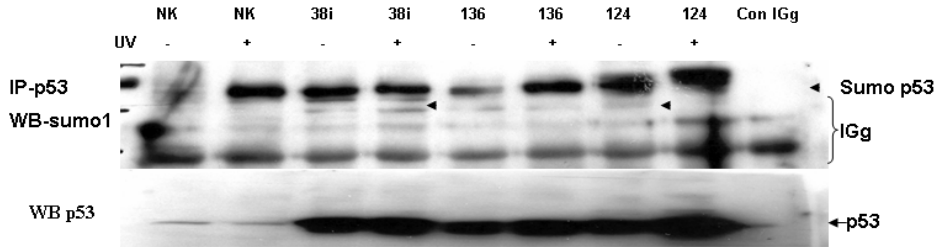


Figure 26. Analysis of p53 sumoylation with and without UVB treatment Cells were exposed with and without UVB for 10 minutes. Then, total cell lysates were prepared and immunopresipitated with p53 monoclonal antibody and immunobloted for sumo-1 with anti-sumo-1 antibody. The same blot was stripped and immunobloted for p53 with polyclonal antibody for checking p53 input.

Our data support the finding of Kwek et al. 2001 that SUMO-1 modification of p53 at lysine 386 may not be essential for p53's cellular localization, transcriptional activation, or growth regulation, at least in HNSCC. Interestingly, we have noticed that some proteins, which are interacting with p53, have 90KD size accumulated in cancer cell lines in comparison with normal keratinocytes. Moreover, this unspecific p53 interacting protein level is increased after UVB treatment (Figure 26). Further analysis is required to understand sumoylation of p53 with sumo2 and 3 involved in wild type p53 accumulation and if it exists lead to rise a 90kD size of p53, which we have noticed in our exprements.

4.6.1.3 HPV positive and negative cancer cell lines accumulated wild p53 is hyper-phosphorylated at serine15 and 392 residues

Stabilization of wild type p53 is normally associated with phosphorylation (Bode and Dong, 2004). To evaluate the phosphorylation status of p53 in human keratinocytes immortalized by HPV38 and other head and Neck cancer harbouring wild type p53, we first determined its protein levels by immunoblotting. p53 is strongly accumulated in HPV38-keratinocytes and HNC-136 and HNC-124 in comparison with primary keratinocytes (Figure 27a). Immunoblot analysis using antibodies specific for various phosphorylated forms of p53 showed a marked increase in phosphorylation of p53 at serines 15, 46 and 392 in HPV38-keratinocytes, HNC-136, and HNC-124, compared with normal keratinocytes (Fig 27a and b).

Figure 27a

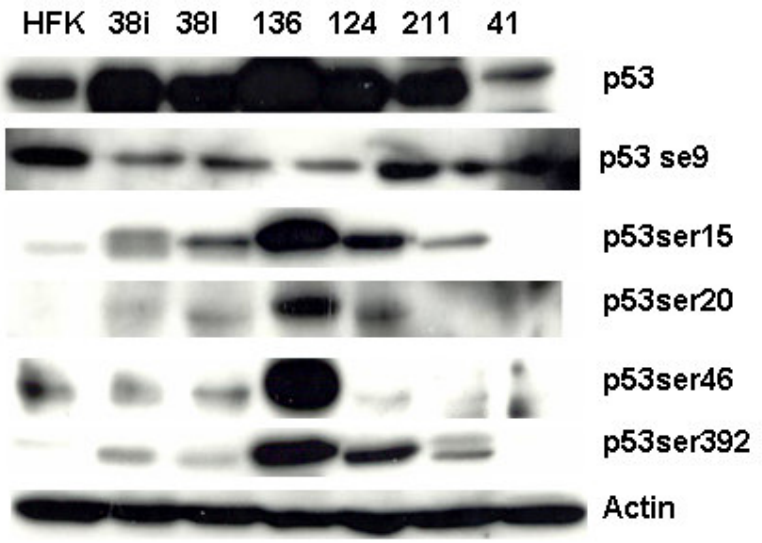


Figure 27a. Status of phosphorylation of p53 in HPV Negative Head and Neck Cancer Cells. Protein extracts of the indicated cells were analyzed by immunoblotting using the indicated antibodies.

In contrast, serine 9 was less phosphorylated in the HPV38, HNC-136 and HNC-124 cells accumulating wild type p53 in comparison with control cells (normal keratinocytes) (Figure 27 a and b).

Figure 27b

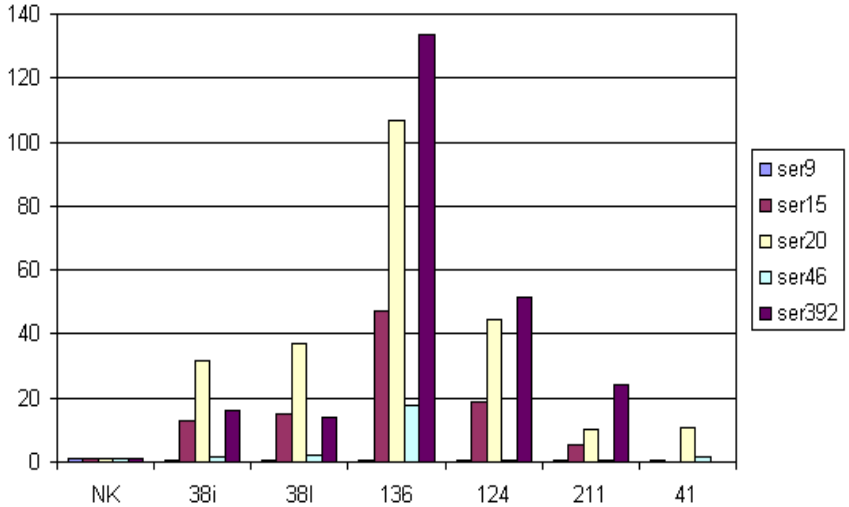


Figure 27b. Characterization of p53 phosphorylation pattern. After normalization of the levels of the different phosphorylated forms of p53 to the signal for total p53, the relative level of p53 phosphorylation in HFK, HPV38 E6/E7(38i and 38I), and other cancer cell lines (HNSCC) were evaluated in comparison with primary keratinocytes.

To exclude the possibility that p53 stabilization might also result from an accidental mutation in the TP53 gene, we sequenced exons 2-10 (plus flanking splice junctions) in the HPV38-keratinocytes, HNC-136 and 124 (these two cell lines were already checked for p53 sequence, see before) and confirmed a wild-type sequence. Moreover, it represents that hyperphosphorylated form of p53 is unable to accumulate MDM2 protein (results 4.6.1.1).

4.6.1.4 Wild-type p53 is accumulated in the nucleus of HPV positive and negative cancer cells

Immunofluorescence staining showed that p53 was distributed in both the cytoplasm and nucleus in control cells (normal human keratinocytes), whereas it was exclusively localized in the nucleus in HPV38-keratinocytes (Figure 28), HNC-124 and -136 accumulating wild type p53 (Figure 29).

Figure 28

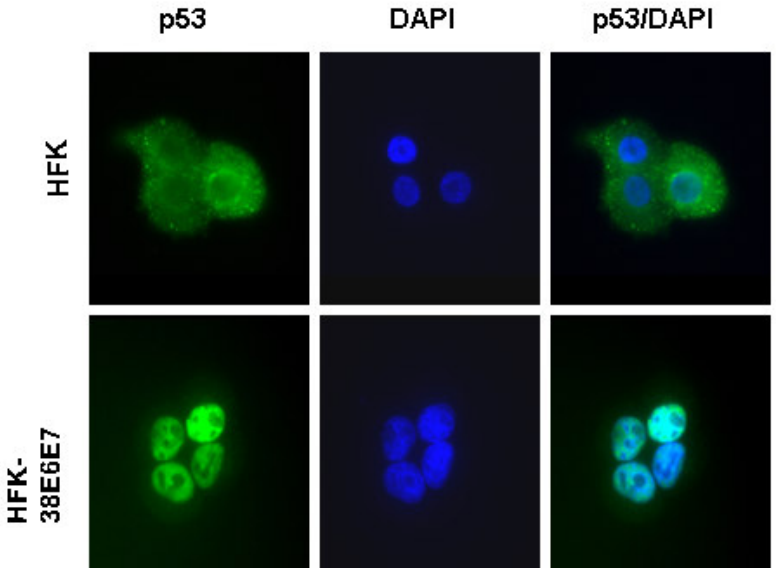


Figure 28. Imunofluorescence showing p53 localization in Primary keratinocytes and in HPV38 keratinocytes.

Figure 29

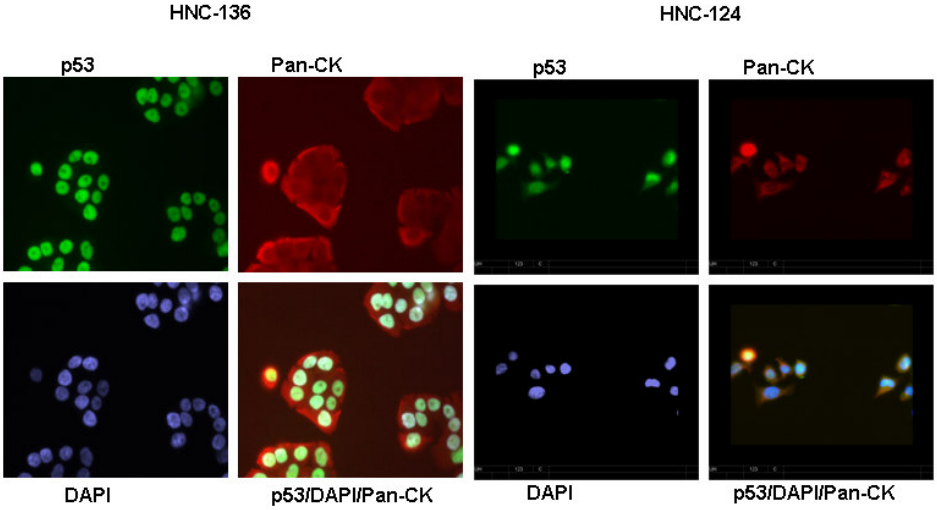


Figure 29. Imunofluorescence showing p53 localisation in HNC136. Monolayer-cultured cell formaldehyde-fixed and stained with antibodies against p53 and/or Cytokeratin with or without DAPI.

Taken together, these data indicates that HPV positive and negative cancer cell lines induce the hyper-phosphorylation of p53 at specific serine residues, resulting in the stabilization and nuclear accumulation of wild type p53 with impaired tumour suppressor functions. Despite the presence of high levels of p53 in the nucleus, HPV38-keratinocyte, HNC-136 and HNC-124 actively proliferate and do not show any sign of apoptosis, indicating that wild type p53 tumor supressor function has been impaired in these cells. Results from molecular characterization of accumulated wild type p53 properties indicate that p53 hypersphosporylation (especially at serine15, 46 and 392) plays a crucial role in wild type p53 accumulation and impairement of tumor supressor functions of p53. Based on these finding, we have further studied the down stream effectors of hyperphosphorylated form of wild type p53 in HPV38 and HNSCC cells.

4.6.2 Accumulated wild type p53 down stream effectors in HPV positive and negative cancer cells

As our HPV positive and negative cancer cell lines were accumulating wild type p53 didn't show any sign of apoptosis and senescence, we were most interested to study its role in cell survival and proliferation pathways. One of the mechanism by which accumulated wild type p53 assist cell survival and proliferation is by specifically activating ΔNp73, already published from our laboratory (Accardi et al. 2006) in HPV38 model. Other than ΔNp73, recent reports suggest that p53 can be involved in different cell survival pathways.

Therefore, we have further analysed those recently indentified p53 regulated cell survival and proliferative protein levels in both HPV38 and HNSCC cells.

4.6.2.1 ΔNp73 protein is up-regulated in HPV positive and negative cancer cell lines accumulate wild-type p53 hyper-phosphorylated at serine15 and 392

It has been shown that isoforms of the p53-related protein, p73, lacking the amino-terminal transcriptional activation Δ(TA) domain, collectively called (delta) isoforms, antagonize the transactivation functions of p53 and p73 (Melino et al. 2002). The isoforms ΔNp73', p73ΔExon2/3 and p73ΔExon2 are generated by alternative splicing at the 5' region of the p73 messenger RNA, whereas the ΔNp73 isoform is expressed by the internal promoter located in intron 3 that contains a p53-responsive element (p53-RE; Levrero et al, 2000; Melino et al, 2002). RT-PCR analysis showed that HPV38-keratinocytes express high levels of the ΔNp73 isofrom (Figure 30). Immunoblot analysis with an anti-p73 antibody detected a protein band with an approximate molecular mass of 60kDa that co-migrated with the ectopically expressed ΔNp73 isoform (Figure 30), confirming the RT-PCR data.

Figure 30

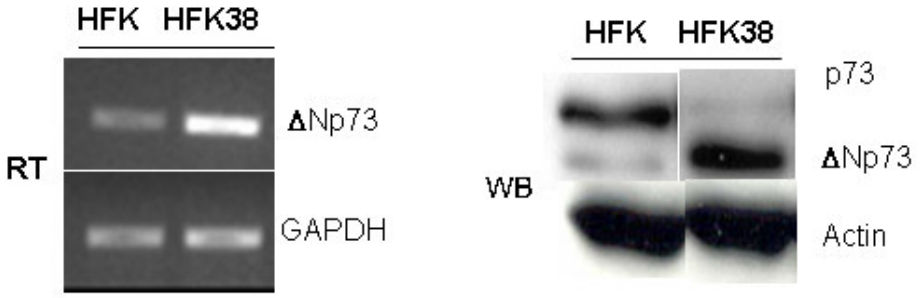


Figure 30. HPV38 E6 and E7 expression promotes ΔNp73 accumulation. (A). Reverse transcription-PCR (RT-PCR) was performed using specific primers for the ΔNp73. (B) ΔNp73α protein is present at high levels in HPV38-keratnocytes.

For understanding this scenario in HPV negative and wild type p53 accumulated HNSCC cancer cell lines, we have analyzed ΔNp73 isofrom in HNC-136 and HNC-124 and found that in these cell lines high levels of ΔNp73 are expressed at protein level (Figure 31)

Figure 31

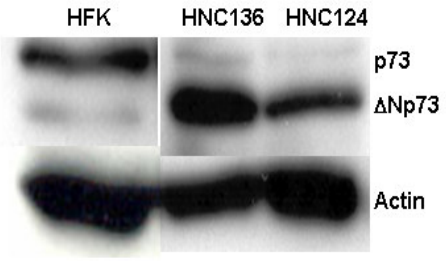


Figure 31. Western blot analysis showed that HNC-136 and HNC-124 express high levels of ΔNp73 isofrom at protein level.

Our data shows that both HPV positive and negative cancer cells modulate stabilization of wild-type p53 by hyper-phosphorylation at serine 15 and 392, which in turn preferentially activate transcription of ΔNp73. We also observed that TAp73 is down-regulated in HPV38-keratinocytes and Head and Neck cancer cell lines (HNC-136 and -124). As ΔNp73 is able to antagonize p53 and p73 transcriptional function, the TAp73 down regulation is probably less relevant than ΔNp73 accumulation. Interestingly, it has been shown that ΔNp73a shows in vitro transforming activities (Petrenko et al, 2003) and is up regulated in many human cancers (Zaika et al, 2002; Concin et al, 2004).

4.6.2.1.1 Inhibition of MEK1 cause down regulation of ΔNp73 in HPV38 and HNC-136

Human p53 has 23 different phosphorylation and dephosphorylation sites and p53 serine residue phosphorylations are correlating with p53 activation scenario. Moreover, our cancer cell lines are proliferating with accumulated wild type p53 hyperphosphorylated at serine15 and serine392, we have decided to analyse possible kinases, which are involved in these phosphorylation. Several serine-thrionine kinases are reported to phosphorylate p53 at several residues and some kinases are redundant in phophorylation of different p53 serine residues. Using specific protein kinase inhibitors, we examined the involvement of protein kinases in p53 phosphorylation and also tried to analyse p53 serine15 and serine 392 phoporylation, important for ΔNp73α protein accumulation. PD98059, a MEK1 kinase inhibitor treatment in our HPV38 keratinocytes and HNC-136 significantly inhibited p53 serine15 and 46 phosphorylation and it led to the down regulation of ΔNp73 (Figure 32).

Figure 32

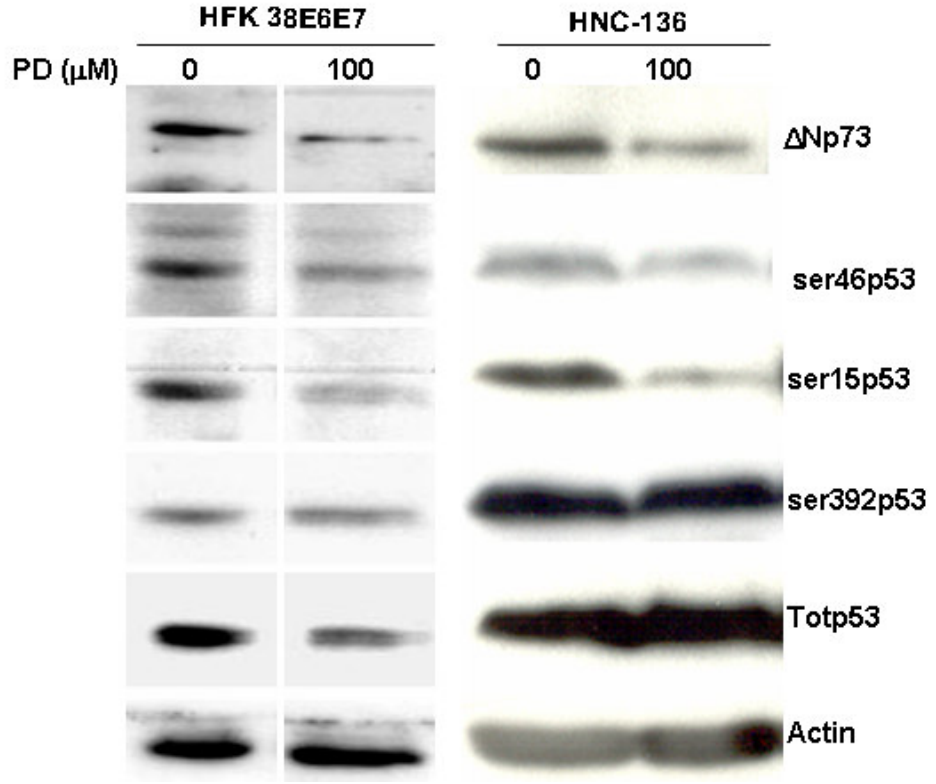


Figure 32. HFK38E6E7 and HNC-136 were treated with the MEK1 inhibitor PD, after 24 hours the cells were collected and analyzed for the indicated proteins by Immunoblot analysis.

Our analysis showed that p53 ser15 and ser46 phosphorylation have been mediated my MEK/MAPK cascade of kinases and these signal trasduction pathway is activated constituently in HPV positive and negative cancer cell lines where wild type p53 is hyperphosphorylated at serine15 residue. Moreover, these phoshorylation influences p53 mediated accumulation of ΔNp73 protein.

4.6.2.2 TIGAR protein status in HPV positive and negative cancer cell lines

Many p53 regulated cell survival genes have recently been reported and among them one is p53-inducible gene named TIGAR (TP53-induced glycolysis and apoptosis regulator). TIGAR expression lowered fructose-2,6-bisphosphate levels in cells, resulting in an inhibition of glycolysis and an overall decrease in intracellular reactive oxygen species (ROS) levels. These functions of TIGAR correlated with an ability to protect cells from ROS-associated apoptosis, and consequently, knockdown of endogenous TIGAR expression sensitized cells to p53-induced death. Expression of TIGAR may therefore modulate the

apoptotic response to p53, allowing survival in the face of mild or transient stress signals that may be reversed or repaired. The decrease of intracellular ROS levels in response to TIGAR may also play a role in the ability of p53 to protect from the accumulation of genomic damage. As our interest was to analyse the role of accumulated wild type p53 in HPV38 and other Head and Neck Cancer cell lines, we checked TIGAR protein level by western blot experiment and found that level of TIGAR protein is higher in Keratinocytes and comparatively less expressed in Head and Squamous cell carcinoma cell lines (Figure 33).

Figure 33

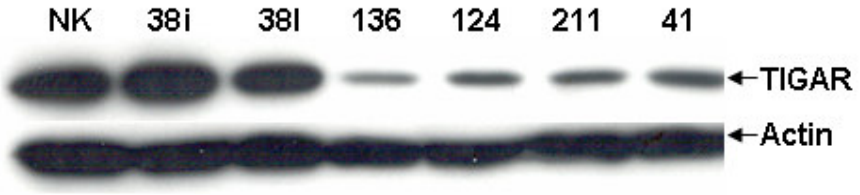


Figure 33. Western blot analysis shows TIGAR protein level is high in Keratinocyte origin in comparison with Head and Neck squamous carcinoma cell lines. Among head and Neck cancer cell lines HNC-136 showed very less level of TIGAR protein.

We didn't find any definite correlation between p53 status and TIGAR expression. Because, among different head and neck cancer cell lines were analyzed HNC-136 was the one accumulated more wild type p53 and very less TIGAR protein level in combarison with other HNC-cell lines. In our study, keratinocytes showed more TIGER protein level indicating that this protein has major cell protection function in keratinocytes from reactive oxygen species (ROS) induced by UV-B radiation.

4.6.2.3 SCO2 expression levels in HPV positive and negative cancer cell lines accumulating wild-type p53

p53 has been shown to induce the expression of the copper transporter SCO2, which is required for the assembly of cytochrome *c* oxidase (Matoba, S. et al. 2006). This allows p53 to enhance oxidative phosphorylation. Conversely, the loss of p53 activity in cells results in a reduction in oxygen consumption. As our cell lines were accumulating wild type p53, we were more interested to analyze whether these cell lines are more depends upon oxidative phosphorylation pathway for high energy demanded for continues cell proliferation. Our analysis found that HPV38 keratinocytes are over expressing SCO2mRNA with respect to control keratinocytes but we found less expression of SCO2mRNA in HNC-136 (Figure 34).

Figure 34

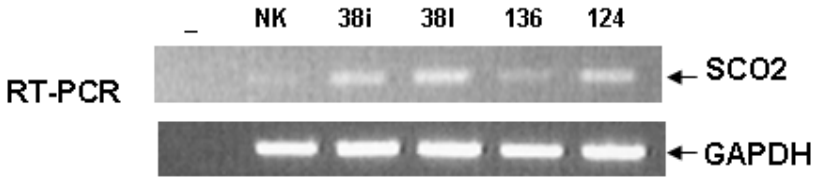


Figure 34. p53-regulated SCO2 gene is unregulated in HPV38-HFKs but we found lower level of SCO2 mRNA in HNC-136

For further conforming our RT-PCR data, we have analyzed SCO2 protein level in our cell lines and found that p53 accumulated HPV38 keratinocytes and Head and Neck cancer cell lines over-express SCO2 at protein levels in comparison with Normal Keratinocytes (Figure 35).

Figure 35

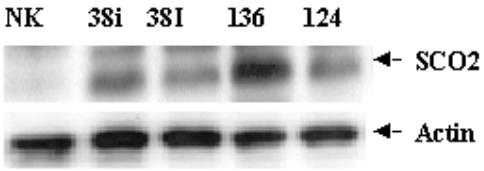


Fig 35. SCO2 protein is over-expressed in HPV38 and Head and Neck cancer cell lines accumulated wild type p53.

In our analysis, we found that HNC-136 expresses comparatively less amount of SCO2mRNA but its protein level is high with respect to other cancer cell lines analyzed. Cells can obtain energy through the oxygen-dependent pathway of oxidative phosphorylation (OXPHOS) and through the oxygen-independent pathway of glycolysis. Since OXPHOS is more efficient in generating ATP than glycolysis, it is recognized that the presence of oxygen results in the activation of OXPHOS and the inhibition of glycolysis (Pasteur effect). The finding that p53 was inactivated in the majority of cancers and that the majority of tumors also exhibit the Warburg effect has stimulated to consider whether p53 may regulate glycolysis. Our results supports the possibility that wild type p53 accumulated cells depend upon oxidative phosphorylation for cell proliferation.

4.6.2.4 p21WAF-1 protein level in HPV positive and negative cells

Next we have analyzed p21waf1 level in our cells accumulating wild type p53. p21waf1 has been shown to mediate the p53-dependent growth arrest induced by DNA-damaging agents. Our analysis showed that p21waf1 is slightly accumulated in HPV38 keratinocytes in comparison with control cells (Figure

36). HNC-136 showed relatively less p21 protein and it may correlate with its hyper proliferative characteristics indicated in Table 3.

Figure 36

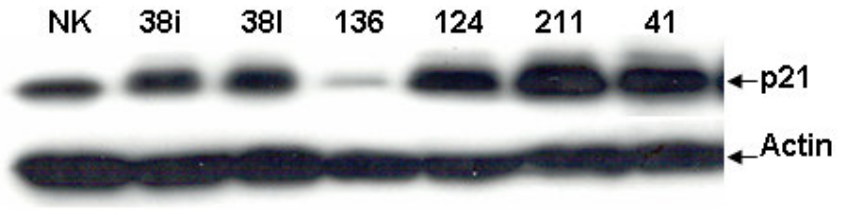


Figure 36. Western blot analysis shows that p21waf1 protein is slightly accumulated in HPV38 keratinocytes but HNC-136 shows comparatively less protein than other cell lines analyzed.

Several functions have been ascribed to p21waf1 that could be involved in this growth arrest. For one, p21waf1 is an efficient inhibitor of cyclin-dependent kinases (CDKs). Moreover, p21waf1 can interact with proliferating cell nuclear antigen (PCNA), and as such inhibit in vitro DNA-replication. Finally, p21waf1 has been reported to inhibit stress-activated protein kinases (SAPKs). In our system p21 level is indespensible to the p53 status. These results revealed a p53-independent role in p21 induction in HNSCC.

4.7 Loss of p53 leads to growth arrest in both HPV38 immortalized keratinocyte and Head and Neck cancer cell lines (HNC-124 and HNC-136)

Our study revealed that wild type p53 hyper-phosphorylated at serine15 and 392 is accumulated in some cancers, both HPV38 positive and negative cells and favouring for ΔNp73 accumulation and cell survival. For confirming these aspects and understanding in more detail the functions of accumulated p53 role in cell survival, we have knockdown p53 by retroviral vector expressing p53-shRNA and performed colony formation assay. We have produced retrovirus contain shRNA against p53 in phoenix cells and infected HPV38, HNC-124 and HNC-136 cells. After one-day infection cells were tripsinised, counted and equally plated for colony formation in a selection medium containing puromycin. As a control we have used (scramble agamy) retrovirus vector alone, which doesn't contain shRNA for p53. After several days of selection visible colonies were formed, and stained with crystal violet and found that p53 shRNA infected plates contain less colonies in comparison with control plates (Figure 37). We have counted the colonies and found around 50% of reduction in cell colonies by plates, which are infected with shp53 scramble agamy in comparison with control scramble agamy (Figure 37a and 37b).

Figure 37a

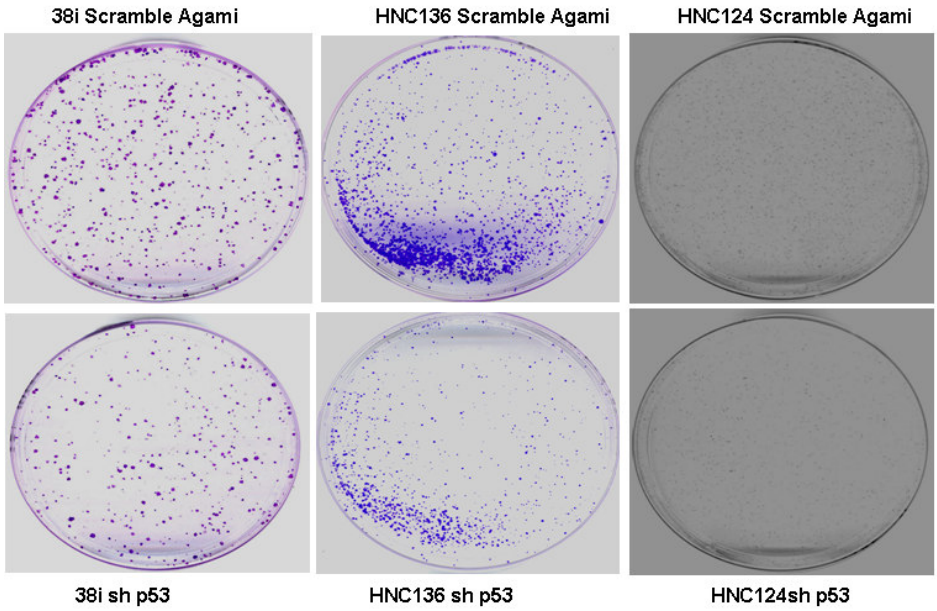


Figure 37a. Both HPV38 and HNC136 were equally plated and infected with scramble (control) and shRNA against p53 by retroviral infection. After complete selection colonies were stained with crystal violet.

Figure 37b

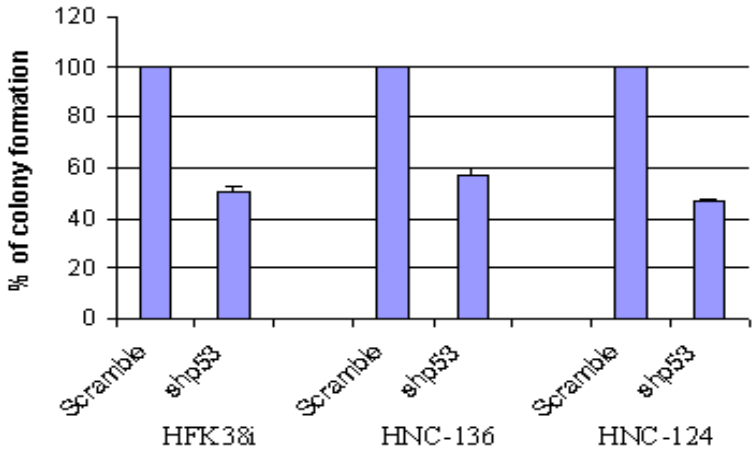


Figure 37b. Inhibition of colony formation in shp53 infected plate in comparison with control plates in percentage.

In colony formation assay, we have used retroviral vector expressing shRNA against p53 and selection with peuromycin. Peuromycin selection made difficult to analyze p53 knock down effects in these cells, other than colony

formation assay. Therefore, we used siRNA against p53 to down regulate p53 expression to analyze its down stream effectors.

4.7.1 p53 knockdown by siRNA induces morphological changes in HPV positive and negative cancer cells

We have performed p53 knockdown by transfection of siRNA against p53 (indicated in material and methods) by fugine reagent. As a control scrable siRNA was used. After 48 hours of tranfection cells were analyzed and collected for RNA and protein analysis. After 48 hours of transfection analysis shown significant difference in cellular phenotypes in p53 siRNA transfected cells in comparison with control (scramble) and shown in Figure 38.

Figure 38

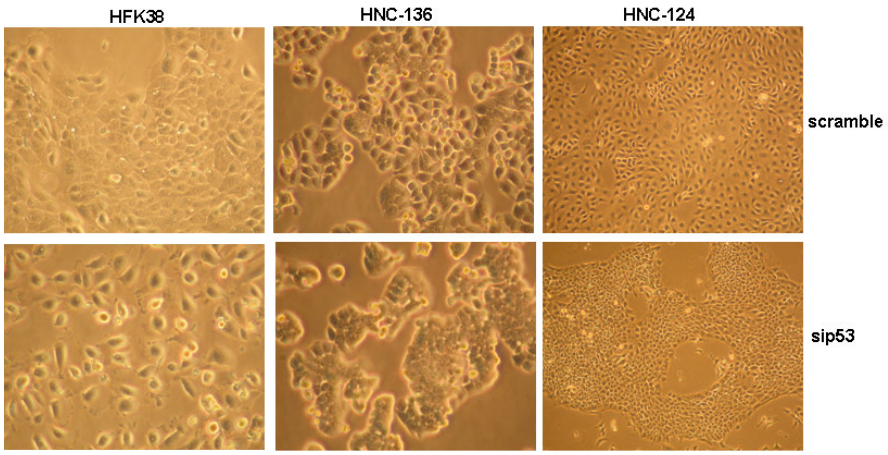


Figure 38. HPV38, HNC-136 and HNC-124 were treated with siRNA against p53. After 48 hours of siRNA treatment against p53, cells show different morphological features in comparison with control (scramble).

After 48 hours of p53 siRNA treatment inhibited cell proliferation and induced contractile cell phenotypes in comparison with scramble. Among the cell lines analyzed, HNC-124 shwed very prominent phenotypic changes after p53 knock down. Figure 39 shows difference in phenotypes after 48 hours of p53 siRNA treatment at different magnifications (Figure 39).

Figure 39

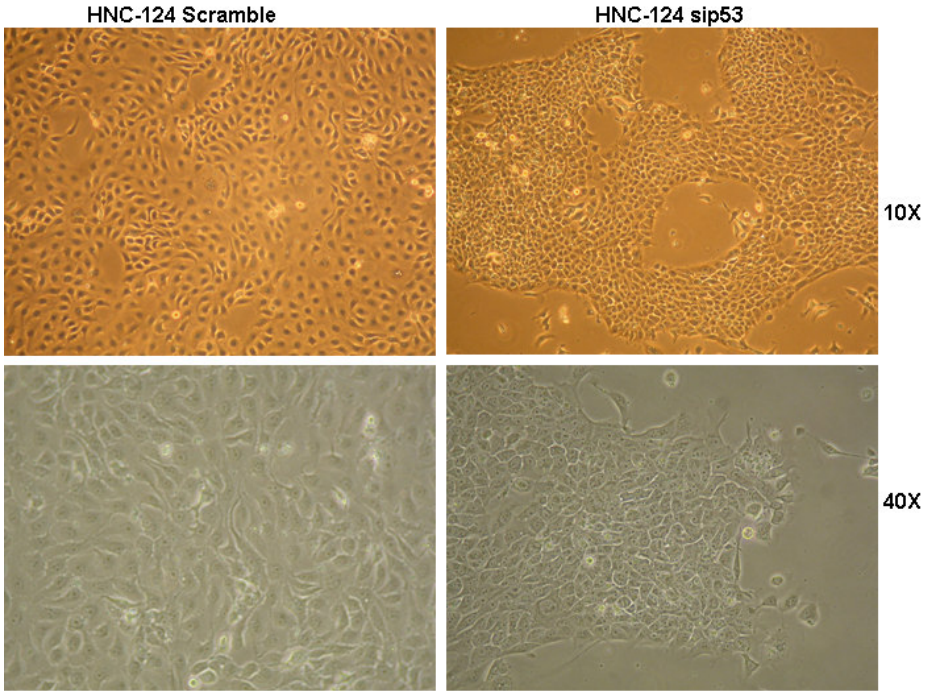


Figure 39. HNC-124 showed more prominent morphological changes after p53 knockdown. After 48 hours of siRNA treatment against p53, HNC-124 cells are shown with different magnifications.

4.7.2 p53 knock-down inhibited cell proliferation in HPV38 and HNSCC cells

We have also performed cell proliferation assay after p53siRNA treatment. After 48 hours of transfection plates were stained with cristal violet and showed that p53 siRNA treated plates present very less cell density in comparison with control plates, where plates became 95% confluent (Figure 40).

Figure 40

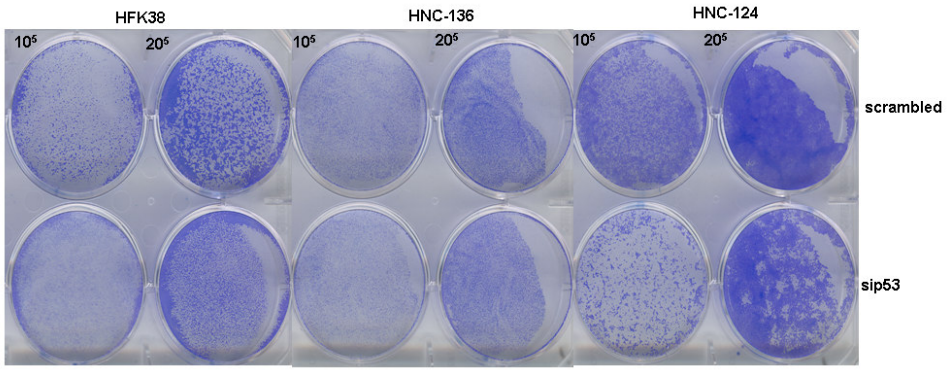


Figure 40. p53 knock down by siRNA inhibited cell proliferation in HFK38, HNC-136 and HNC-124. Cells were plated in a six well plate at different cell numbers and treated with siRNA against p53 and as a control scramble siRNA. After 48 hours of treatment wells were stained with crystal violet and showed that siRNA treatment reduced cell growth in HFK38, HNC-136 and HNC-124 in comparison with control (scramble).

In Figure 41, we show that silencing of p53 by siRNA down-regulate p53 levels in all cancer cell lines understudy. HFK38 shown more down regulation of p53 protein (around 80%) than HNC-136 and HNC-124 (Figure 41). Next, we determined p53 short interfering RNA (siRNA) mediated inhibition of cell proliferation due to apoptosis by checking PARP cleavage (Figure 41).

Figure 41

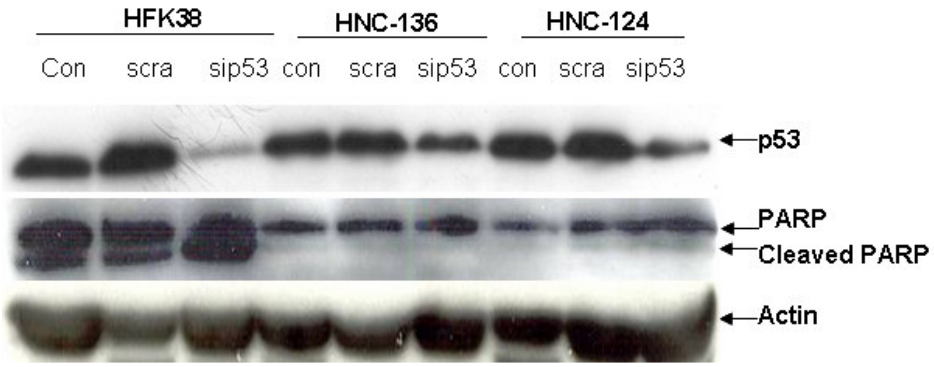


Figure 41. Characterization of p53 knock down induced apoptosis. After 48 hours of post-transfection of HPV38- keratinocytes and Head and Neck Cancer cell lines (HNC-136 and HNC-124) with indicated short interfering RNAs (siRNAs) protein extracts were analyzed by immunoblotting using the indicated antibodies.

We have found cleaved PARP in p53 siRNA treated HFK38 cells but not in head and neck cancer cells. This may due to the high p53 down regulation in this cells after p53 siRNA treatment in comparison with HNSCC cells.

4.7.3 p53 Knock-down induced senescence-associated β-galactosidase activity

p53 knockdown inhibited cell proleferation in all cell lines analyzed and slight apoptosis induction only in HFK38. But we have found significant cell morphology pattern like scenescence in all cell lines, therefore we have analyzed for senescence induction in these cell lines after 48 hours of p53 siRNA treatment (Figure 42a).

Figure 42a

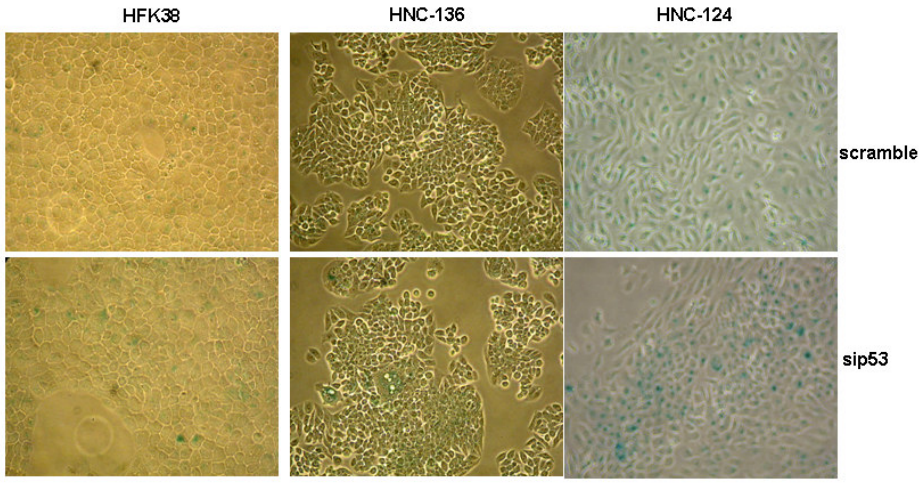


Figure 42a. p53 knock down induced senescence in HPV positive and negative cancer cells. Cells were plated in a six well plate at different cell numbers and treated with siRNA against p53 and as a control scramble siRNA. After 48 hours of treatment wells were stained for beta galactosidase activity (senescence detection) and showed that siRNA treatment induced senescence in HFK38, HNC-136 and HNC-124 in comparison with controls (scramble).

Our senescence analysis revealed that p53 knowck down induced slight increase in beta galatosidase activity in HFK38, HNC-136 and more in HNC-124 (Figure 42b).

Figure 42b

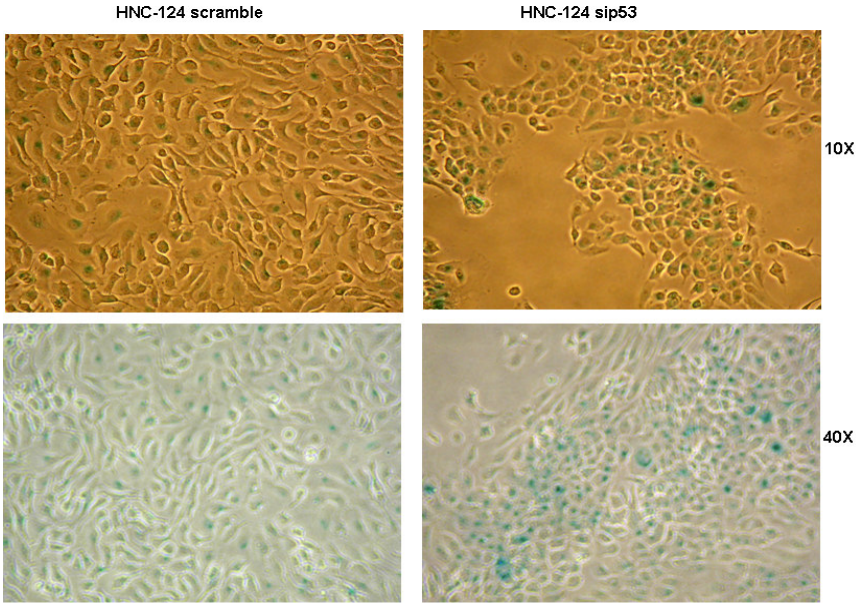


Figure 42b. HNC-124 has shown prominant senesence induction after p53 knock down. After 48 hours of siRNA treatment against p53, senescence detection was performed with senescence detection kit from cell signalling by manufactures protocol. HNC-124 cells have shown prominant senescence associated beta galatosidase activity and shown with different magnifications.

Our results are novel, in the sense that in some cancers, where wild type p53 is not mutated instead wild type p53 is accumulated and favors cell proliferation. Moreover, p53 knock down inhibited cell proliferation and induced senescence. Cellular senescence has been theorized to oppose neoplastic transformation triggered by activation of oncogenic pathways *in vitro (*Lowe and Cepero, 2004; Serrano and Blasco, 2001). Here, we propose that accumulated wild type p53 protein in some cancers suppresses oncogenes induced senescence, insted of assisting cell proliferation, at least in some cancers. This may be the consequence of wild type p53 knock down induced senesece in our HPV positive and negative cancer cells. Future experiments are called for to understand how wild type p53 by passes senesence and assists cell proliferation.

4.7.4 p53 knock-down in HPV38 and Head and Neck Cancer cell lines down-regulate ΔNp73 expression

Next, we determined whether down-regulation of p53 by short interfering RNA (siRNA) decreases ΔNp73 levels in our HPV positive and negative cancer cell lines. Figure 43 shows that silencing of p53 expression correlated with down regulation of ΔNp73.

Figure 43

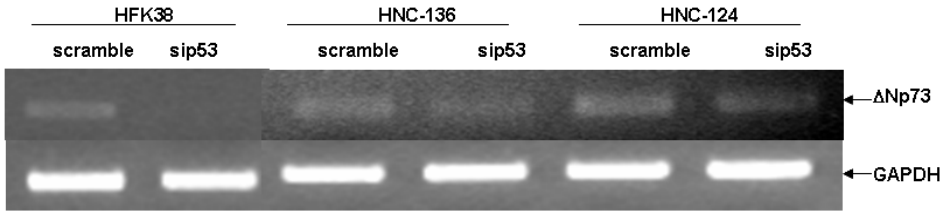


Figure 43. ΔNp73 expression is mediated by p53. After transfection of HPV38- keratinocytes and Head and Neck Cancer cell lines (HNC-136 and HNC-124) with indicated short interfering RNAs (siRNAs), ΔNp73 expression levels were determined by reverse transcription–PCR (RT–PCR; right panel), respectively. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HFK, human foreskin keratinocyte.

Our study identifies the preferential role of hyper-phosphorylation mediated accumulation of wild type p53 in ΔNp73 up-regulation. But ΔNp73 up-regulation alone assist cell survival and cell proliferation of HPV positive and negative cancer cells by inhibiting senescence; this aspect has to be further analyzed. For further understand molecular signalling pathways involved in cellular senescence after p53 knockdown, we have analysed several targets shown in Figure 44.

Figure 44

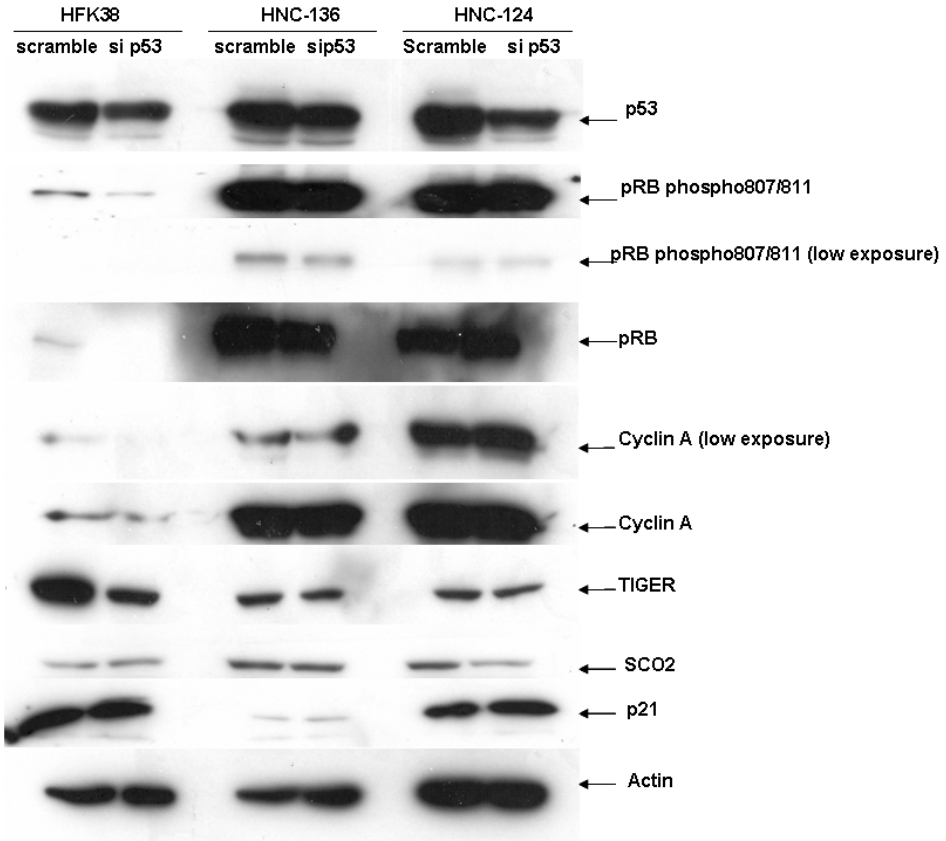


Figure 44. Characterization of p53 knock down induced downstream effects. After 48 hours of post- transfection of HPV38- keratinocytes and Head and Neck Cancer cell lines (HNC-136 and HNC-124) with indicated short interfering RNAs (siRNAs) protein extracts of the indicated cells were analyzed by immunoblotting using the indicated antibodies.

Other than ΔNp73 down regulation some cell survival and proleferative regulatores like pRB, CyclinA, TIGAR and SCO2 also altered after p53 knockdown. p53 down-regulation inhibited pRB phosphorylation at serine807/811 and it may corresponds to slight pRB accumulation in HFK38 and HNC-136. TIGAR has shown down regulated in HFK38 and 136 but at higher level in HFK38 and it may be the reason of slight apoptosis induction in HFK38 after p53 knock down. Cyclin-A has down regulated only in HFK38 after p53 knockdown but p21 levels remained constant in all cell lines analysed. In this experiment p53 down regulation is more prominent in HFK38 but very less in HNSCC cells. We need futher obtimization for p53 knock down in HNSCC cells to analyze in detail the down stream effectors and this can be done only by using stable siRNAs. Due to time constraint, we could not perform those experiments and include in this thesis.

4.8 Literally known Breast and Colon Cancer cell lines also accumulate wild type p53 protein hyperphosphorylated at serine15 and 392

For expanding our study, we have analysed literally known breast (breast cancer cell lines with wild type p53 were HCC-1937, Cal-51, and MCF-7) and colon (colon cancer cell lines with wild type p53 were HCT-116, LS-174T, Co-115, and TC-7) cancer cell lines harbouring wild type p53 and find that some of them show accumulated wild type p53 hyperphosphorylated at serine15 and 392 (Figure 45). Moreover, p53 accumulated cells contain very less amount of MDM2 protein (Figure 45)

Figure 45

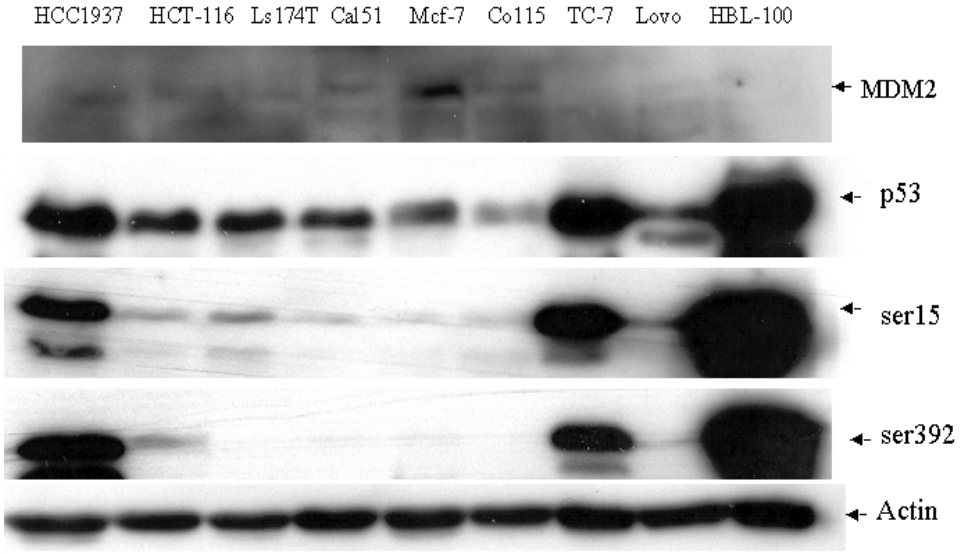


Figure 45. Western blot shows that wild type p53 is accumulated and hyperphosphorylated at serine15 and 392 in some of the breast and colon cancer cell lines. Serine15 and 392 hyperphosphorylated and accumulated cell lines were less expressing MDM2 at protein level.

Our data show that in absence of p53 mutation, some percentage of cancer cells accumulate wild type p53 hyperphosphorylated at serine15 and 392 and it may be significant for cancer cell survival. Due to time constraint we couldn't perform same p53 knockdown experiments to analyse this accumulated p53 role in cell proliferation and whether p53 knowck down induces senescence in these cell lines. For detailed understanding further p53 cell survival pathways have to be characterised in these cell lines.

Part II

4.9 Anti-EGFR Therapy In Head and Neck Cancer In-vitro Model

Head and neck cancer (HNC) accounts for about 5% of all cancers world-wide, and the overall survival period is very short as the cancer often metastasises in the lymph nodes of the throat. The failure of conventional chemotherapy, which does not increase the overall survival rate of patients, and the goal of identifying new biological targets for anticancer therapy have stimulated the study of the molecular mechanisms of HNSCC progression. Among the promising new cellular targets, epidermal growth factor receptor (EGFR) seems to have a prominent role since it has been recognized as a prognostic factor in head and neck cancer. One of the most promising is the utilisation of tyrosine kinase (TK) inhibitors, targeting epidermal growth factor receptor (EGFR) in HNSCC. It has recently been suggested that gefitinib (ZD1839; Iressa), an orally active, selective EGFR tyrosine kinase (TK) inhibitor can being utilized in head and neck cancer therapy (www.clinicaltrials.gov). As an in-vitro model of Head and Neck cancer, we aimed to molecularly characterize our large panel of HNSCC cell lines for EGFR and its related receptors expression and down stream effectors to use gefitinib as an anti-cancer therapy. Moreover, we have compared the targets analyzed, “p53 family network” and gefitinib effectiveness.

4.9.1 Major TK receptors EGFR (ErbB1), ErbB2, and VEGFR2 status in head and neck cancer cell lines

The expression level of TK receptors, EGFR, ErbB2, and VEGFR-2 protein status, determined in our newly established HNSCC cell lines are shown in Figure 46.

Figure 46

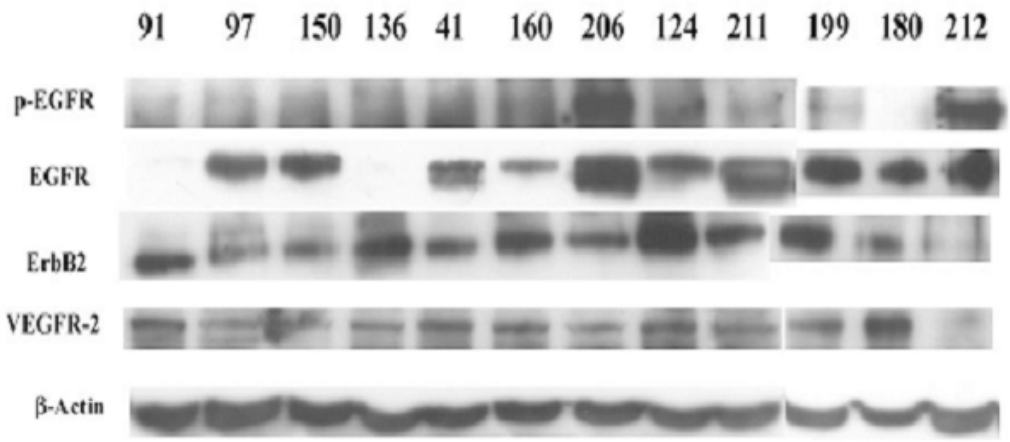


Figure 46. Major TK receptors (EGFR, ERB2 and VGFR-2) status in Head and Neck Cancer Cell Lines

Higher levels of EGFR were observed in HNC-211, -199, and -206 cells whereas it was undetectable in HNC-91 and -136. Conversely, only HNC-206 and -212 showed detectable levels of p-EGFR. The other two TK receptors, ErbB2 and VEGFR-2 were ubiquitously expressed in all cell lines (Figure 46). Moreover, activated Akt has been reported to stabilize MDM2 and lead to p53 protein checkup (Feng et al. 2004). We choose to analyse TK receptors, especially EGFR pathway downstream effectors, as total and phosphorylated form, trying the possibility to suggest a biological therapy with EGFR inhibitors.

4.9.2 Status of major EGFR down stream effectors in HNSCC cell lines

Once EGF receptor is activated it lead to the activation of its down stream effectors, mainly Akt and Erk1/2 phosphorylation. In our all cell lines we found comparatively similar expression pattern of Akt and Erk1/2, conversely, p-Akt and p-Erk1/2 varied among cell lines characterised (Figure 47).

Figure 47

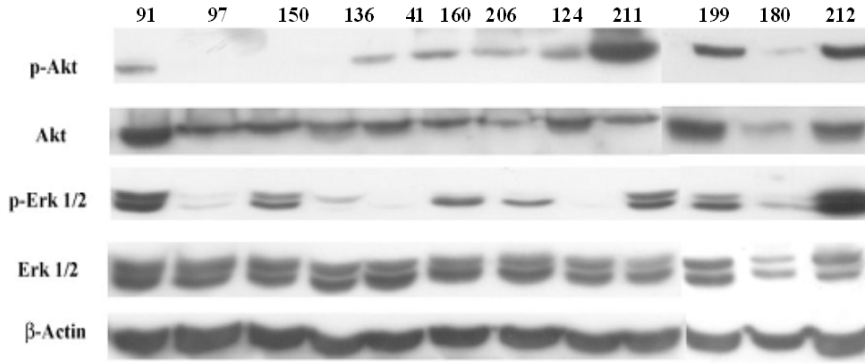


Figure 47. EGFR down stream effectors in Head and Neck Cancer Cell lines.

Very low or undetectable amount of p-Akt was observed in HNC-97, -150, -136 mean times HNC-211 showed high level of this phosphorylated protein (Figure 47). Undetectable amount of p-Erk1/2 was observed in HNC-41 and –124, mean time HNC-91 and 212 showed high level of this phosphorylated protein (Figure 47). Interestingly the tumour suppressor, PTEN which acts upstream Akt activation, was expressed ubiquitously in all lines (Figure 48).

Figure 48

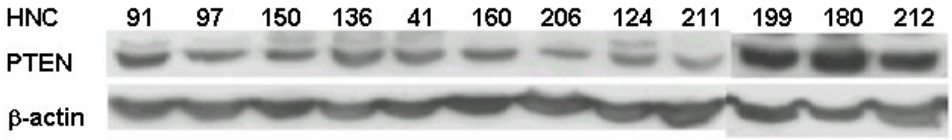


Figure 48. The tumor suppressor gene PTEN protein level in head and neck cancer cell lines and it's ubiquitously expressed and not correlated with EGFR down stream effector activator p-AKT.

PTEN was reported as epigenetically silent in many tumours but our analysis showed it is ubiquitously expressed in head and neck cancer cell lines and not correlated with p-Akt activation (Fig.48). Our results supports findings by Henderson (Henderson et al. 1998), who reported a low percentage of inactivated form of PTEN in head and neck cancer.

4.9.3 Influence of p53 family network on EGFR signal transduction pathway

Wild type and mutated p53 can differentially transactivate the EGFR promoter by binding to a response element present in the EGFR promoter. However, EGFR promoter sequence requirements for transactivation by wild type p53

and mutated p53 are different (Ludes-Meyers et al. 2006). In addition to p53, its closely related homologue p63 has also been shown to regulate the EGFR promoter, through multiple encoded proteins (TAp63, ΔNp63). In specific, the p63 isoform, TAp63gamma, represses EGFR expression by directly interacting with Sp1 and impairing Sp1 binding to the target DNA (Nishi et al. 2001). In this perspective it was worthy to analyse p53 family tumor suppressors influence in EGFR signal transduction pathway. We found the existance of correlation only between ΔNp63 and EGFR expression, both reported as oncogenes in the progression of Head and Neck cancer. Infact among twelve cell lines analysed, only two were devoid of both EGFR and ΔNp63 (HNC-91 and 136) while in the others both proteins were co-expressed.

4.10 Anti-EGFR drug, gefitinib effects on head and neck cancer cell lines

In this study, we tested gefitinib effectiveness in a broad panel of 12 newly established HNSCC cell lines, investigating its ability to reduce cell growth, to induce apoptosis and to modulate cell cycle and various EGFR pathway-related targets. Moreover, p53 family proteins influence on the efficacy of EGFR inhibitor, gefitinib, was investigated.

4.10.1 Gefitinib IC_{50} values in HNSCC cell lines

The capability of gefitinib to inhibit cell growth was determined by incubating each HNSCC cell line with various drug concentrations for 3 days, and the results are reported as IC50 values in Table 7.

Table 7

Cell Lines	IC-50 (μM) ± S.D.
HNC-211	0.064±0.01
HNC-180	0.71±0.12
HNC-97	1.9±0.9
HNC-124	1.9±1.2
HNC-160	2.2±1.1
HNC-212	4.22±1.8
HNC-91	5.3±2.0
HNC-41	5.4±1.6
HNC-206	8.3±2.4
HNC-136	9.3±1.9
HNC-199	25±3.3
HNC-150	33±2.7

Table 7. IC50 value for Gefitinib in each Head and Neck Cancer Cell lines.

Out of all cell lines, HNC-211 was the most sensitive of the drug, with IC50 of 64 nM, and the most resistant ones were HNC-150 and -199 (IC50 of 25 and 33 μM, respectively). The other cell lines showed 10- to 300-fold greater resistance to the drug than HNC-211. The validity of our *in vitro* model for a complete analysis of gefitinib activity in HNSCC, was warranted by such a significant cell line-specific drug variability.

4.10.2 Gefitinib-dependent cell cycle Inhibition

The high variability of gefitinib IC50 values among cell lines suggested to investigate drug activity in each cell line. In particular, the ability of gefitinib to modulate cell cycle progression was determined. A three-days drug exposure induced a slight blockage at G0/G1 phase in the cell cycle, with a maximum effect on HNC-206 inducing G0/G1 phase accumulated cells around ~22% (Table 8).

Table 8

	No gefitinib				+ gefitinib 3 days
Cell lines	**DNA index**	**G0/G1 phase**	**S phase**	**G2/M phase**	**increase in G0/GI phase %**
HNC-211	**1.58**	**63.63**	**19.26**	**17.08**	**9**
HNC-180	**1.75**	**47.64**	**33.63**	**18.73**	**17**
HNC-97	**1.48**	**73.95**	**4.81**	**21.24**	**1**
HNC-124	**1.41**	**54.95**	**19.77**	**25.28**	**7**
HNC-160	**1.13**	**66.62**	**13.81**	**19.56**	**1**
HNC-212	**1.67**	**60.65**	**4.06**	**16.29**	**9**
HNC-91	**1.76**	**73.48**	**6.63**	**19.89**	**4**
HNC-41	**1.54**	**65.45**	**22.05**	**17.50**	**0**
HNC-206	**1.36**	**51.77**	**30.07**	**18.17**	**22**
HNC-136	**1.48**	**63.01**	**6.93**	**30.06**	**5**
HNC-199	**1.37**	**60.70**	**16.21**	**23.08**	**7**
HNC-150	**1.43**	**74.60**	**5.99**	**19.41**	**0**

Table 8. Gefitinib induced cell cycle inhibition. After 3 days Gefitinib treatment cells were stained with propedium iodide and analyzed by FACS.

Our analysis revealed that gefitinib induced cell cycle inhibition and that is not the main mechanism of its antiproliferative effects because in such large panel of head and neck cancer cell lines, HNC-206 and HNC-180 only showed significant cell cycle arrest at G0/G1 phase of cell cycle.

4.10.3 Gefitinib induced apoptosis in HNSCC cells

Gefitinib effectiveness to induce apoptosis in Head and Neck cancer cell lines has also been determined by annexinV-FITC and propidium iodide using the Apo-Target kit (BD transduction). All cell lines were treated with Gefitinib at IC50 concentrations for 1 to 3-days and apoptosis induction was analysed by flow cytometry analysis. Only in five cell lines (HNC-211; -124; -136; -199 and -150) we found an increase (10-20%) of the Annexin V positive cells. These results indicate that there was not a direct correlation between apoptosis induction and drug sensitivity because our most sensitive and resistant cell lines, HNC-211 and -150, respectively showed apoptosis at IC-50 concentrations of Gefitinib treatment. In our twelve cell lines analysed, HNC-211 and HNC-180 showed comparatively more apoptotic cells during increasing time interval of drug exposure and shown in Figure 49.

Figure 49

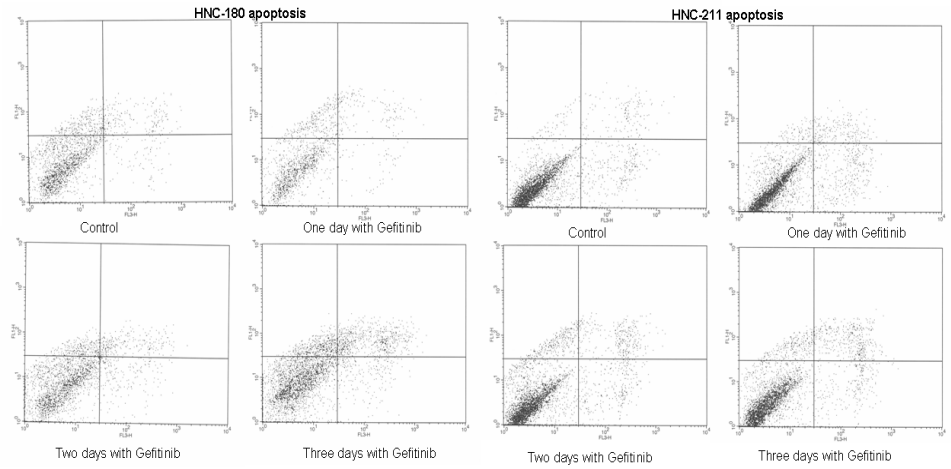


Figure 49. HNC-211 and HNC-180 cell lines were treated with Gefitinib at IC-50 concentrations for different time periods and analysed for apoptosis detection by Anexin-V method. Figure shows that percentage of apoptosis increases with increasing time intervals.

Gefitinib was able to induce apoptosis after 3 days of exposure mainly in the most resistant cell lines. This was probably due to the high drug concentration rather than specificity of action. Notably, this TK inhibitor also induced apoptosis in two sensitive cell lines, suggesting that its ability to initiate the apoptotic process could be cell line-specific. This idea is supported by the evidence that in the resistant cells, apoptosis increased in a time-dependent manner leading to the hypothesis that the mechanism of drug saturation could be involved.

4.10.4 Gefitinib inhibits phosphorylation of p-EGFR, p-Akt and p-Erk1/2 in HNSCC cells

The drug-dependent modulation of EGFR and its downstream effectors, Akt and Erk1/2, has also been investigated. Each cell line was treated with gefitinib at IC50 concentration for three days and the modulation of total and phosphorylated forms of EGFR, Erk1/2 and Akt was analysed. Gefitinib did not modulate the expression of EGFR, Akt and Erk1/2 (data not shown). Only HNC-212 and -206 cells showed detectable levels of p-EGFR expression and a 3-day gefitinib treatment showed 80% of residual EGFR in the phosphorylated form (Figure 50b and c). p-Akt was completely abolished by gefitinib in four cell lines, HNC-41,-124, -97 and -212, and variously reduced in the remainder, conversely p-Erk1/2 was reduced to a lesser extent with the exception of HCN-180 and -160, as reported in Figure 50 a, b and c, and summarised in Table 9.

Figure 50a

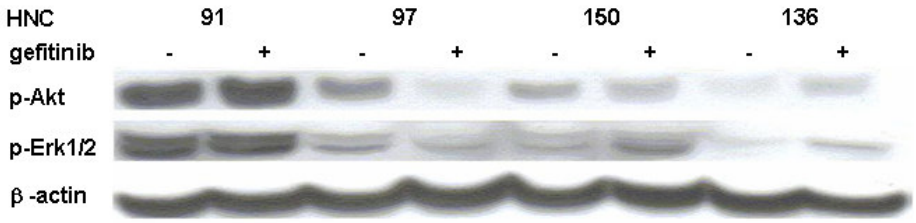


Figure 50b

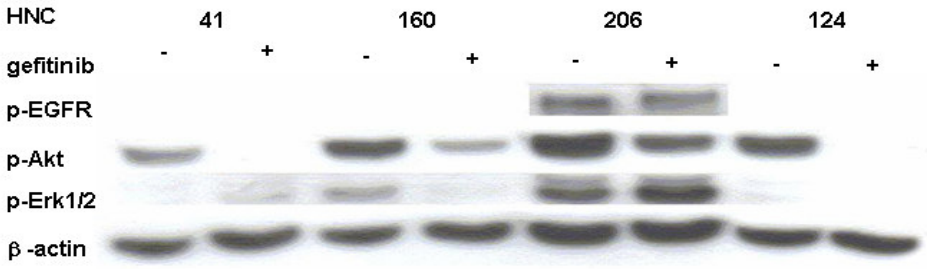


Figure 50c

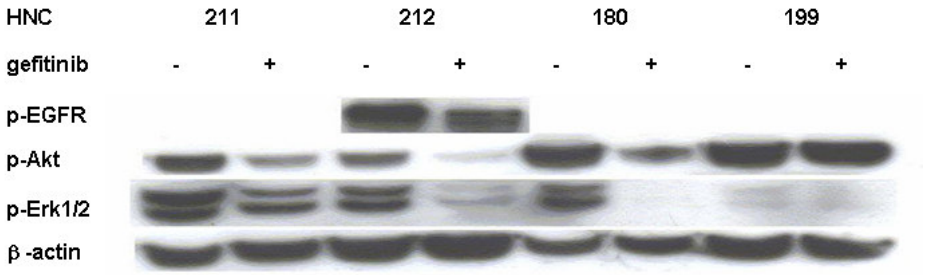


Figure 50 a, b and c. EGFR pathway modulation by gefitinib. HNSCC cells were incubated with gefitinib at IC50 concentration and the protein extracts were analysed by immunoprecipitation and/or Western blotting. In Western blot analysis, the amount of the different targets was determined using monoclonal- or polyclonal- specific antibodies and ß-actin was used to normalise the values. p-EGFR was clearly detectable only in HCN-212 and HCN-206.

Table 9

Cell Lines	IC-50 (μM) ± S.D.	Increase in G0/G1 phase %	Apoptosis	p-EGFR expression %	p-Akt expression %	p-Erk1/2 expression %
HNC-211	**0.064±0.01**	**9**	**+**	**ND**	**20**	**40**
HNC-180	**0.71±0.12**	**17**	**-**	**ND**	**10**	**0**
HNC-97	**1.9±0.9**	**1**	**-**	**ND**	**0**	**50**
HNC-124	**1.9±1.2**	**7**	**+**	**ND**	**0**	**ND**
HNC-160	**2.2±1.1**	**1**	**-**	**ND**	**10**	**0**
HNC-212	**4.22±1.8**	**9**	**-**	**80**	**0**	**10**
HNC-91	**5.3±2.0**	**4**	**-**	**ND**	**100**	**100**
HNC-41	**5.4±1.6**	**0**	**-**	**ND**	**0**	**ND**
HNC-206	**8.3±2.4**	**22**	**-**	**80**	**30**	**100**
HNC-136	**9.3±1.9**	**5**	**+**	**ND**	**ND**	**ND**
HNC-199	**25±3.3**	**7**	**+**	**ND**	**100**	**100**
HNC-150	**33±2.7**	**0**	**+**	**ND**	**30**	**100**

Table 9. Gefitinib effectiveness in HNSCC cell lines

Analysis of the modulation of EGFR signal transduction pathways by gefitinib showed that this drug, after three days of exposure, did not seem to inhibit EGFR phosphorylation completely. However, based on our previous evidence (Giannelli et al. 2004), we hypothesised that gefitinib inhibited EGFR activation early on, while after a prolonged exposure (3 days), the phosphorylated form of the receptor recovered its baseline level. Analysis of the ability of gefitinib to reduce the activation of two main signal transduction pathways, PI3K/Akt/mTor and Ras/MAPK, evidenced a selective action on the first of these two signaling pathways.

4.10.5 EGFR mutation analysis in HNSCC cell lines

Recently, EGFR mutations have been reported to determine gefitinib sensitivity and resistance, we have sequenced EGFR kinase domain coding exons (exons 18 to 21) and carboxyl terminal exons 27 and 28. Mutational analysis, performed in the region of the EGFR gene coding for the tyrosine kinase domain and in the ATPase domain showed that all HNSCC cell lines were wild type. However, this characterisation, utilising primers designed about 150 nucleotides downstream and upstream exonic region, evidenced intronic variants (IVS) (Table 10). Our sequencing analysis found some missense mutations in HNC-180, -199, -211 and -136 along with intron variant sequences listed in Table 10.

Table 10

Cell Lines	Ex 18	Ex 19	Ex 20	Ex 21	Ex 27	Ex 28
HCN 180	wt	WT	G787A G>A Gln>Gln	WT	WT	WT
HCN 199	wt	WT	G787A G>A Gln>Gln	WT	WT	WT
HCN 206	wt	WT	WT	WT	WT	WT
HCN 211	IVS 163924 C>A; IVS 164250 C>T	IVS 164996 G>A	G787A G>A Gln>Gln; IVS 171340 T>C	WT	WT	WT
HCN 41	IVS 159715 C>A omoz	WT	IVS 167131 T>C;	WT	WT Intron delA pos 188573	WT
HCN 91	wt	IVS A>G rs2017000	IVS 167131 T>C;	WT	WT	WT
HCN 97	wt	WT	WT	WT	WT	WT
HCN 124	wt	WT	WT	WT	WT	WT
HCN 136	wt	WT	G787A G>A Gln>Gln	WT	WT	WT
HCN 150	wt	WT	WT	WT	WT	WT
HCN 160	wt	wt	WT	WT	WT	WT
HCN 212	wt	wt	WT	WT	WT	WT

Table 10. EGFR kinase domain and carboxyl terminal exon and flanking intron sequence alterations. Table legends IVS: intron variants, WT: wild type.

HCN-211 presented IVS17-104 C>A and IVS18+103 C>T intronic variants while HCN-41 cell lines only IVS17-104 C>A. The intron 19 presented two different types of intronic variants: IVS19+96 A>G in HCN-91 and HCN-211 and IVS 19-60 in HCN-41, -91, -199 and -211. However, these intronic variants seeme not interfere in EGFR expression. Based on evidence that in lung cancer, certain mutations in the region of the EGFR gene coding for the ATPase and tyrosine kinase domains are related to drug sensitivity, we analysed our 12 cell lines for EGFR mutation status, confirming the data of Puhringer-Oppermann, suggesting that these mutations are not relevant in determining gefitinib response in oral cancer (Puhringer-Oppermann et al. 2007).

4.10.6 Correlation of p53 family proteins influence in gefitinib sensitivity

Cellular sensitivity to the growth inhibitory effects of gefitinib remained unchanged, irrespective of p53 and its family proteins status. In fact our molecular characterisation results concerning the mutations and protein level of p53, the mRNA and protein level of p73 (isoforms ΔNp73 and TAp73) and p63 (isoforms ΔNp63 and TA p63) among twelve HNSCC cell lines were not correlated to gefitinib sensitivity. Moreover, p53 protein levels did not influence gefitinib sensitivity in our panel of head and neck cancer cell lines. Table11 summarises the results of p53 and its homologous correlation with Gefitinib drug sensitivity.

Table 11

Cell Lines	IC-50 (μ M) ± S.D.	p53 Status				p63 RT-PCR		p63 Western		p73 RT-PCR		p73 Western	
		Exon	Codon base	Amino acid change	Protein expression	ΔN	TA	ΔN	TA	ΔN	TA	ΔN	TA
41	5.4±1.6	WT (4-9)			-	-+++	+	+++	+++	+	+	++	+++
91	5.3±2.0	WT (4-9)			-	-	-	-	-	++	-	Nd	Nd
97	1.9±0.9	Exon 5 Exon 7	180 GAG-AAG 248 CGG-TGG	Glu-Lys Arg-Trp	+	+	-	+	+	++	-	+	-
124	1.9±1.2	WT (4-9)			++	+++	-	+++	+++	+++	-	++	-
136	9.3±1.9	WT (4-9)			+++	-	+	-	-	++	++	+++	-
150	33±2.7	Exon 5	135 TGC-TAC	Cys-Tyr	+	+++	+	++	+++	++	-	++	-
160	2.2±1.8	Exon 6	213 CGA-CGG	Arg-Arg	-	+++	+	+	++	++	-	Nd	Nd
180	0.71±0.12	Exon 6	220 TAT-TGT	Tyr-Cys	+	+++	++	Nd	Nd	-	-	+	++
199	25±3.3	Exon 5	135 TGC-TTC	Cys-Phe	+	+++	-	-	-	++	-	+	-
206	8.3±2.4	Exon 5	134 TTT-TCT	Phe-Ser	+	+++	++	+	++	-	-	+	++
211	0.064±0.01	Exon 6	177-183 Del 5 codons	In frame	+	+++	+	+++	+++	++	-	++	-
212	4.22±1.8	Exon 6 Exon 9	196 CGA-CCA 331 CAG-.AG	Arg-Pro Frameshift	++	+	-	-	-	-	-	++	-

Table 11. Summary, p53 and its homologous are not directly influencing gefitinib drug sensitivity in a large panel of Head and Neck cancer cell lines.

Although our cell characterisation did not identify any valid predictive factor for gefitinib effectiveness, the comparison between p53 family and EGFR expression suggested, for the first time, a correlation between the ΔNp63 isoform and EGFR expression level. Transcription factors of the p53 family are mainly considered to be tumour suppressors, even though recent evidence demonstrated opposite functions for the p63 isoforms, ΔNp63 and TAp63, promoting cell proliferation and mimicking tumour suppressor p53, respectively (Foschini et al. 2004). In our study, two of the HNSCC cell lines did not express either ΔNp63 or EGFR, while the remainder co-expressed both proteins, suggesting a direct correlation between EGFR and ΔNp63 expression in the development of head and neck squamous cell carcinomas.

4.11 Chemoresistance tests to head and neck cancer cell lines

Resistance to chemoterapy remains a major obstacle to the successful management of many human cancers, especially in HNSCC. More specifically, the cellular resistance to anti-cancer agents arises through failure of p53 family member signalling. Chemoresistance tests were performed to investigate whether different levels of p53, p63, p73 and their isoforms differently affect resistance to chemotherapy. All the twelve cell lines were analysed for chemoresistance and the drugs chosen for the test were *gembitabine* and *cisplatin*. Cell lines reacted in various different ways to the administration of the two drugs. Most of the cancer cells showed a high resistance to the treatment with gemcitabine, in particular the HNC 136; while only four cell lines appeared to be sensitive, with highest level of mortality found in the 124. For cisplatin test, the cell lines presenting a high resistance were the 212 and even in this case the 136, while the most sensitive were the 206 and again the 124. The results obtained were contradictory, for example, the two cell lines with the highest (HNC 136) and the lowest (HNC 124) resistance to gemcitabine both harbour a wild type p53 (Table 12).

Table 12

Cell Lines	p63 RT- PCR		p63 Western		p73 RT-PCR		p73 Western		p53 Status	
	ΔN	TA	ΔN	TA	ΔN	TA	ΔN	TA		
41	+++	+	+++	+++	+	+	++	+++	- WT	
91	-	-	-	-	++	-	Nd	Nd	- WT	
97	+	-	+	+	++	-	+	-	+	
124	+++	-	+++	+++	+++	-	++	-	++ WT	S
136	-	+	-	-	++	++	+++	-	+++ WT	R
150	+++	+	++	+++	++	-	++	-	+	
160	+++	+	+	++	++	-	Nd	Nd	-	
180	+++	++	Nd	Nd	-	-	+	++	+	
199	+++	-	-	-	++	-	+	-	+	
206	+++	++	+	++	-	-	+	++	+	S
211	+++	+	+++	+++	++	-	++	-	+	
212	+	-	-	-	-	-	++	-	++	R

Table 12. Summary of the results. The results obtained by RT on p73 and ΔNp73, p63 and ΔNp63, by western blot analysis on p53 and the data from the chemoresistance tests are listed and compared in the above table.

Again we didn't see correlation between resistance to chemotherapeutic drugs and the state of p53, neither with the level of expression of the other p53 homologues. We assumed that other p53 indipendent pathways may be involved in chemioresistance and apoptotic prevention in head and neck cancer cell lines. Further work is necessary to understand the complex regulatory network generated by multiple p73 and p63 isoforms, their expression profiles, the interactions among themselves and the resulting differentials in their activities. In addition, such large spectrum of malignant cells needs to be surveyed to determine whether consistent protein expression pattern could be identify when drug sensitivity or resistance would be predicted.

4.11.1 Elevated expression of ABCG2 protien in HNSCC cell lines

To justify the high resistance of Head and Neck cancer to some chemotherapeutic drugs during treatment, we determined the expression level of one drug efflux pump protein ABCG2 in our in vitro panel (Figure 51).

Figure 51

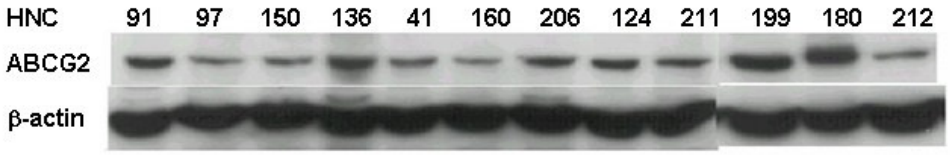


Figure 51. Western blot analysis shows ABCG2 protein level in the twelve head and neck cancer cell lines.

Interestingly, ABCG2 was expressed in all our HNSCC cell lines with very high expression in HNC-180, -199 and 136 in comparison with other cell lines were analysed and these may be one of the reasons of high drug resistance of HNC-136 to cisplatin and gemcitabine. Furthermore our study revealed that cell sensitivity to growth inhibitory effects of ZD 1839 remained unchanged irrespective of ABCG2 status.

5. CONCLUSIONS

Molecular characterization of newly established cancer cell lines are very valuable source for understanding molecular mechanism of cancerogenesis and demonstrating the efficacy of various anti-cancer therapy in Head and Neck Squamous Cell Carcinoma (HNSCC). In this study, we have molecularly characterized 12 novel HNSCC cell lines for understanding p53 family netwok, efficacy of anti-EGFR therapy and p53 family network influence in gefitinib effectivness. p53 family network is considered the skeleton of cellular signalling pathway to co-ordinate both internal and external signals received by a single cell to determine its ultimate fate. Generaly, p53 has been considerd as a tumor suppresson due to its ability to induce cell cycle inhibition and/or apoptosis depends upon the interrupted signal received by a cell. Majority of cancers arises in presence of an inactive p53 (mainly by mutation) and consequently introduction of wild type p53 in those cells can induce apoptosis and/or senescence, it depends upon the cellular system. Here, we found out a new scenario where there are some cells acquiring immortalization in presence of wild type p53 protein and in those cells p53 has been modified in such a way to assit cell survival and proliferation. This hypothesis is confermed by HPV38 mediated immortalization process, where wild type p53 protein is accumulated in favor of cell survival and proliferation. For extending this phenomenon in other cancers, we have molecularly characterized a large panel of head and neck cancer cell lines and revealed that this mechanism is not restricted to HPV38 mediated immortalization process but also exists in other sporadic cancers. Our detailed analysis in both HPV positive and negative cancer cells for p53 showed that accumulation of wild type p53 is correlated with its hyperphosphorylation at serine15 and 392 residues. Moreover, this led to the over-expression of DNp73 in these cells. Using specific protein kinase inhibitors, we examined the involvement of protein kinases in p53 phosphorylation at serine 15 and 392 residues. PD98059, a MEK1 kinase inhibitor treatment in our HPV38 keratinocytes and HNC-136 cells significantly inhibited p53 serine15 phosphorylation and it led to the down regulation of DNp73. Our analysis for other p53 post-translational modifications showed less influence in wild type p53 accumulation in the nucleus and activation of cell survival and/or proliferative genes like DNp73. To further understand functions of hyperphosphorylated and accumulated wild type p53 in HPV38 and HNSCC cells, we have knocked down p53 by siRNA. Interestingly p53 knock down inhibited cell proliferation and induced senescence in these cells. This was associated with down regulation of p53 mediated cell survival and/or proliferative genes, examples are deltaNp73 and TIGER. Other cell survival and/or proliferative genes, specifically under control of this hyperphosphorylated and accumulated wilde type p53 in cancer cells, have yet to be characterized.

As part of a translational cancer research work, we have analyzed feasibility of using target oriented drug like gefitinib in head and neck cancer. We have characterized our Head and Neck in vitro panel extensively, with the aim of identifying predictive factors for gefitinib effectiveness. Recently improved understanding of the pathogenesis of human head and neck squamous cell carcinoma (HNSCC) have led to the development of new molecular based therapeutic strategies; one of the most promising is the utilization of tyrosine kinase (TK) inhibitors, targeting epidermal growth factor receptor (EGFR). Our molecular analysis revealed that all cell lines were free of human papilloma virus infection, four harbored wild-type p53, with two of them accumulating wild type p53, and all of them variously expressed other two p53 family members, p63, p73 and its isoforms. The comparison between the targets analyzed and gefitinib effectiveness evidenced the absence of a clear relationship, allowing to exclude them as predictive factors for gefitinib effectiveness. Our results confirmed the in vitro efficacy of an anti-EGFR approach, but other targets than those analyzed in my thesis should be characterized in order to identify valid predictive factors for gefitinib utilization in HNSCC

Resistance to chemoterapy remains a major obstacle to the successful management of many human cancers and p53 and p73 seem to have an important role in mediating the activity of anti-cancer drugs. More specifically, the cellular resistance to anti-cancer agents arise through failure of p53 family signalling network. Chemoresistance tests were performed to investigate whether different levels of p53, p63, p73 and their isoforms differently affect resistance to chemotherapy. All twelve cell lines were analysed for chemoresistance and the drugs chosen for the test were gembitabine and cisplatin. The cell lines reacted in various different ways to the administration of drugs. Most of the cancer cells showed a high resistance to the treatment with gemcitabine, in particular the HNC 136; while only four appeared to be sensitive, with highest level of mortality found in the 124. For the cisplatin test, the cell lines presenting a high resistance were the 212 and even in this case the 136, while the most sensitive were the 206 and again the 124. The results obtained were contradictory, for example, the two cell lines with the highest (HNC 136) and the lowest (HNC 124) resistance to gemcitabine both harbour a wild type p53. Again we didn't see any correlation between resistance to chemotherapic and the state of p53, neither with the level of expression of other p53 homologues. We assume that other p53 indipendent pathways may be involved in chemoresistance and apoptotic prevention in head and neck cancer cell lines. It may include ABCG2 because ABCG2 was expressed in all our HNSCC cell lines with very high expression in HNC-180, -199 and 136 in comparison with other cell lines analysed and these may be one of the reason of HNC-136 high drug resistance to cisplatin and gemcitabine.

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