MOLECULAR AND EXPERIMENTAL BIOLOGY IN MEDICINE

p53: TUMOUR PROGRESSION AND THERAPY MARKER

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Abstract: *TP53* is tumour suppressor gene located on chromosome region 17p13. It codes protein p53 that is responsible for cell growth regulation through inhibition of the cell cycle or promotion of apoptosis. Stress caused by DNA damage, different aberrant growth signals from the environment and expression of various oncogenes influence p53 expression and activation in both normal and cancer cells. Tumours with different cell of origin, tumours developed in different localisations and the ones present in different stages of progression often harbour p53 aberration. In chronic lymphocytic leukaemia *TP53* mutation and deletion have an impact on therapy response and overall response. In myeloid neoplasms p53 can be involved in disease development mechanism, but in aberrant form it can also contribute to the progression of otherwise favourable form of the disease. Itôs most known role is the one in colorectal carcinoma where it represents final step of malignant neoplasm development ï transition from adenoma to carcinoma. This article gives a review of p53 function in normal and neoplastic cells with special emphasis on its usefulness as a tumour marker.

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INTRODUCTION

TP53, the most frequently mutated gene in human cancers was at first described as an oncogene. In 1979, six groups of investigators independently showed that its product, protein p53, binds to the large T-antigen in SV40 infected cells and forms complex that stimulates cell growth. Since that finding was consistent with the high levels of protein p53 found in many cancers it fitted very well with the idea that *TP53* was an oncogene. Later on it was shown that the wild type *TP53* gene introduced into normal cell in culture could transform it into tumour cell.[1] Based on this discovery, the search for a tumour suppressor gene on chromosome region 17p, revealed a small region containing *TP53* gene.[2] In order to test whether *TP53* is a tumour suppressor gene, ñtwo-hitò test according to Knudsonôs hypothesis proposing the theory for tumour development was performed.[3] According to this theory ñtwo-hitò test can distinguish whether some mutant gene is an oncogene or a tumour suppressor gene based on knowledge that tumour suppressor gene has to have both alleles mutated while oncogene is altered when only one allele is aberrant. The test showed that both alleles of the p53 gene were changed, and that was a proof that p53 was indeed a tumour suppressor gene. Today, p53 is known as a tumour suppressor gene that codes for protein involved in many mechanisms important for tumour development and progression.

p53-MEDIATED CELL MECHANISMS

TP53 is located on chromosome 17 region p13 and represents the most frequent target for genetic alterations in human cancers. Protein p53 regulates cell

growth in healthy cells either by inhibition of the cell cycle or by promotion of apoptosis.[1]

Function of wild-type p53 is regulated through the negative feedback loop that includes MDM2 protein. When p53 is needed in the cell that is damaged or in the stress, it is expressed and activated by phosphorylation.[1, 4] If there is no need for p53 in the cell, prior to the phosphorylation, it interacts with MDM2. MDM2 is a negative regulator that mediates an ubiquitin-mediated degradation of p53: it stimulates the addition of ubiquitin groups to the carboxyl end of p53 and consequently its degradation. On the other hand, activated p53 itself can induce MDM2 expression by binding to the regulatory region of *MDM2* gene (Figure 1). This well-regulated activation loop can be affected by stress caused by DNA damage, different aberrant growth signals from the environment and expression of various oncogenes.[1] DNA damage activation of p53 is dependent on different protein kinases. The two major kinases are ATM (for ataxia telangiectasia mutated) and CHK2. ATM is stimulated by double-stranded breaks in DNA while CHK2 is in turn stimulated by ATM.[5] These DNA damage inducible kinases can phosphorylate p53 residues on its amino end thus prevent its binding to MDM2 and MDM4.[4, 6] In that way p53 is active and inhibits cell cycle, enabling necessary time for DNA repair mechanisms. The second pathway of p53 activation is triggered by expression of some oncogenes, e.g. *RAS* and *MYC*.[7] This response is primarily mediated through the antagonism of p53-MDM2 interaction by tumour suppressor $p14^{ARF}$. Inactivation of different tumour suppressor genes also activate p53.[6] The third pathway of p53 inactivation involves kinase ATR (for ataxia telangiectasia-related) and casein kinase II. This pathway is mainly induced by chemotherapeutic drugs, UV-light and inhibitors of protein kinases.[5] All pathways that stabilize p53 in the cell inhibit its degradation (Figure 2).

But, an increased level of p53 in the cell is not sufficient for activation of genes that lead to cell death or inhibit the cell division. Conformational changes of the p53, like addition or removal of chemical groups such as phosphate, acetyl, glycosyl, ribose and ubiquitin, are required as well. For binding to specific DNA sequences acetylation of lysine residues or phosphorylation of serine near the carboxyl end of p53 is crucial chemical modification(s). On the other hand a conformational change of p53 on its amino end, by adding mainly phosphate groups, affects its ability to be degraded by ubiquitin-mediated proteolysis. In this way, in normal cells p53 levels are maintained at low level by proteosomal degradation.[1, 7]

p53 NETWORKS

Activation of p53 induces a network of genes in p53 pathway that respond to both intrinsic and extrinsic stress signals. These stress signals have influence on cellular homeostatic mechanisms involved in monitoring of DNA replication, cell cycle, genome stability and cell division.[8] There are various cancer-related signals that activate protein p53: DNA damage, hypoxia, spindle damage, heat or cold shock, nitric oxide associated with tissue inflammation, depletion of ribonucleoside triphosphates and ribosome biogenesis.[4] Protein p53 is mostly modified by phosphorylation and acetylation although the mechanism of posttranslational modifications in response to stress by kinases and histone acetyltransferases still remains unsolved.[5] These protein modifications alter the p53 in two ways. One way is the increase of protein p53 half-life which further increases its concentration in the cell. The other way is ability of p53 to bind specific DNA sequences and promote and/or enhance the transcription of genes regulated by those DNA sequences. Activation of p53 in response to different stress types has one thing in common: disruption of efficient DNA replication which leads to enhanced mutation rate or chromosomal abnormalities during the cell division.

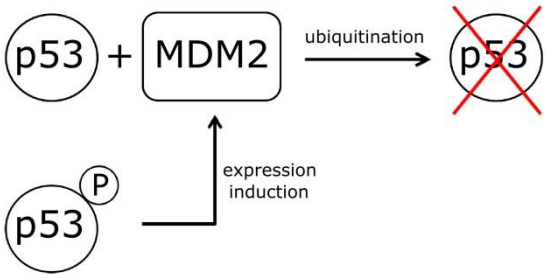

Figure 1. p53 activation loop

Once p53 is activated it binds to a specific DNA sequences, called p53-responsive elements that are found in the first or second intron of a target gene on its 5ô end. The genes in p53 network initiate pathways of cell cycle arrest, cell senescence or apoptosis. Programme for p53-mediated G1 arrest include major player p21 that inhibits cyclin-dependent kinase. The p53-mediated G2 arrest depends on phosphatase that acts upon cyclin B-CDC2 kinase which is in normal condition essential for G2/ M transition. Additionally, there are number of genes directly regulated by p53 that contribute to the cell apoptosis by enhancing the secretion of cytochrome c into the cytoplasm from the mitochondria. Cytochrome c then interacts with APAF-1 to initiate protease cascade, important for activation of caspase-9 and caspase-3 which leads to intrinsic apoptosis pathway. On the other hand, extrinsic pathway involves the activity of caspase-8 and caspase-3 and also induces apoptosis.[6]

TP53 IN TUMORIGENESIS

Protein p53 does not function normally in most human cancers. The critical step in tumorigenesis is

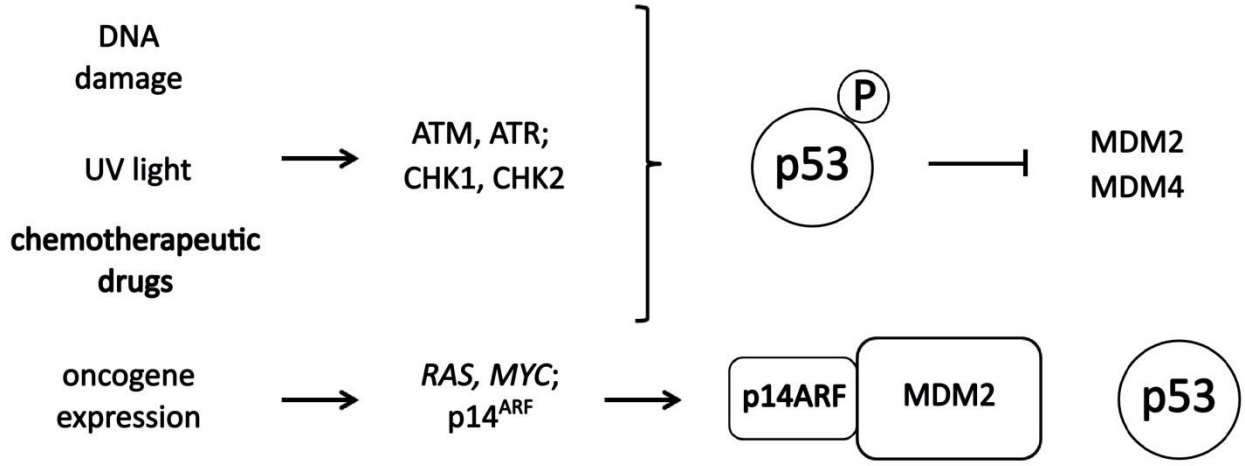

Figure 2. Stress induced activation of p53 network.

inactivation of protein p53, mostly by mutations. But, whether TP53 mutations are the initial steps of malignant transformation or its consequence is still an open question.[9] Since the key role of *TP53* is a trigger of cell cycle arrest or cell death, oncogenic stress can cause different mutations and chromosomal abnormalities involving *TP53* and its pathway and enable tumour cells to survive.[10] In about half of the tumours p53 is inactivated directly by mutations, mostly substitutions or deletions. In other tumours p53 can be inactivated indirectly by viruses or alterations in genes that interact with p53 pathway. *TP53* mutations occur in almost every type of cancer although at different rates, from 10% in haematopoietic cancers to almost 100% in ovary cancers9. In most cancers one allele of *TP53* is altered by substitution while other copy is often deleted from the cell.[4, 5]

TP53 encodes 393aa protein. The promotor of this gene lacks TATA box and has various binding-sites for transcription factors.[11] Most mutations of *TP53* were predominantly found in DNA-binding domain in exons 4-9. In that area there are 6 Ăhot spotsñ mutations at amino acid positions 175, 245, 248, 249, 273 and 282 (Figure 3).[7, 9, 12]

There are a number of p53-induced target genes involved in the processes important for tumour suppression such as apoptosis, cell cycle arrest, DNA repair and senescence.

In a heterozygote genotype which is frequently transient, *TP53* mutations are followed by loss of heterozygosity during tumour progression. Loss of heterozygosity is seen at p53 locus with wild type and mutant allele in which wild type allele becomes deleted or mutated. Most *TP53* mutations are missense mutations caused by substitutions. The majority of these mutations (80%) are found in DNA-binding domain.[8] It seems that mutations in DNA-binding motifs could be also associated with gain-of-function mechanism referring to acquisition of the oncogenic capability of mutant p53 protein.[9, 13] Some p53 mutants are able to trans-activate different genes (for example proto-oncogene c-*MYC*), activation of which can result in growth-promoting phenotypes and drug resistance.[11] Some p53 mutants have ability to bind and inactivate members of its own family like p63 and p73. Their inhibition is considered a key mechanism for mutant p53 gain-of-function.[13]

TP53 GENE MUTATION AND del(17p) IN CHRONIC LYMPHOCYTIC LEUKAEMIA

Chronic lymphocytic leukaemia (CLL) is a good example of a malignant disease in which *TP53* mutation and/or deletion are routinely assessed and have a detrimental impact on therapy response and overall response.[10]

Chronic lymphocytic leukaemia (CLL) is malignant B-cell proliferation. Neoplastic cells, morphologically small lymphocytes with round nucleus and scant cytoplasm, have immunophenotype CD19+/CD5+/CD23+ and they count for more than $5x10^9$ of monoclonal cells in peripheral blood. CLL is usually described as Ăchronic lymphocytic leukaemia/small lymphocytic lymphomañ, because of known non-leukemic cases that show the same morphology and immunophenotype as leukemic ones. In the last decade, entity named monoclonal B-lymphocytosis was recognized and often associated with CLL. It is a clonal B-cell expansion of cells that often have the same immunophenotype as CLL, but it is not certain if it is a predisposition to leukaemia.[14] Such diversity of morphologically similar B-cell proliferations as well as clinically highly variable presentation, course and outcome of the classical CLL suggested that CLL is not homogenous, indolent disease as it was believed to be for a long time. One unifying feature of all CLL subtypes is their cell of origin ï tumour cells arise from mature, antigen-experienced B-cells. However, genetic profiling confirmed heterogeneity of disease on genomic level and postulated different subtypes that are characterized

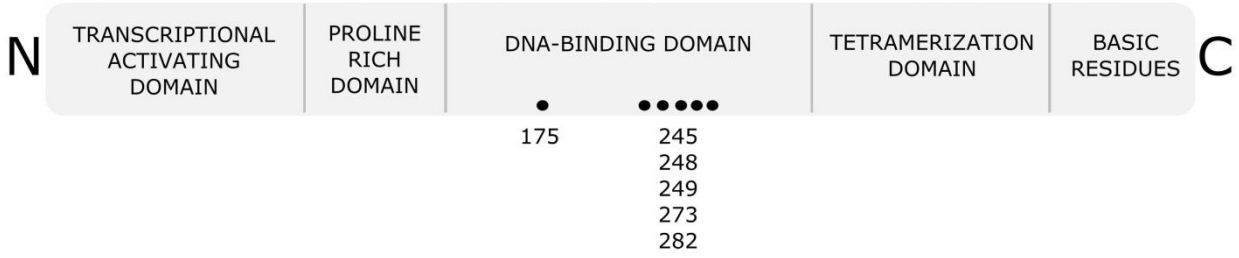

Figure 3. Regions of p53 protein. Dots represent hot-spots mutations in DNA-binding domain.

by genetic aberrations linked with the clinical course of the disease.[14] There are more than 13,000 somatic mutations in protein-coding regions, and around 950 copy number alterations in CLL genome. Only 36 recurrently mutated genes were detected, although single specific gene aberration that would mark this disease cannot be determined.[14, 15] The most significant recurrent aberrations (deletions and/or mutations), due to their association with impaired survival and therapy response, are the ones of *TP53* gene. Dohner et al. found chromosomal aberrations in ~80% of all studied CLL cases.[16] They recorded 17p deletion (deletion of region containing *TP53*) in only 7% of cases, but also showed the association of this cytogenetic abnormality with the shortest median survival time, more advanced disease and shortest median treatment-free interval when compared with other cytogenetic abnormalities they studied. In the same study they presented usage of newly designed set of DNA probes for interphase fluorescent *in situ* hybridization that enables detection of genomic aberrations with clinical relevance in CLL. Soon after, this set became a part of routinely performed standard protocol for evaluation of cytogenetic aberrations at the diagnosis for patients with CLL.[16] *TP53* mutations in CLL were found a decade earlier when its exons 5-8 were sequenced and/or analysed by single-strand conformation polymorphism.[17-19] In one of those studies cytogenetic analysis in comparison with sequencing showed that *TP53* mutation was present in cases with del 17p, while in cases without del 17p mutation could not be found suggesting the relevance of p53 loss of tumour suppressor activity for development of disease.[18] Moreover, *TP53* mutations were associated with the advanced clinical stages and poor outcome.[18, 19] In following years it was generally accepted that *TP53* mutations accompany del 17p. However, in 2008 Zenz et al. studied *TP53* mutations in exons 2-11 by direct sequencing and denaturing high performance liquid chromatography along with fluorescent *in situ* hybridization for detection of del 17p.[20] They showed that although in around 80% of p53 aberrant cases one allele harbours mutation while other is missing due to del 17p, there is around 5% of CLL patients with *TP53* mutation and without del 17p. In this study of 126 cases both monoallelic and biallelic aberrations of p53 were associated with shorter survival.[20] Similar studies confirmed that p53 inactivation in most CLL cases includes mutation in one allele and deletion of the other, but also pointed out the negative impact of sole monoallelic *TP53* mutation on the survival.[21- 23] Those studies were conducted on larger patient cohorts than the ones in 90ôs, and were also able to suggest that p53 mutations might existed in a subclone of tumour cells that expand after the treatment. Being heterogeneous disease, CLL tumour mass in some cases can have small subclone that harbours p53 mutation which is undetectable at the time of the diagnosis. Those cells will not respond to standard therapy protocols, while other subclones will be suppressed by it. In this way, after the initial treatment, surviving p53-harbouring subclone will expand and change the behaviour of the disease and patient status. Moreover, *TP53* aberrations were from the beginning associated with therapy response. Mutations, and later *TP53* deletion, were recognized as markers for non-responsiveness to the therapy with alkylating agents or purine analogues.[19, 24-26] Soon it became evident that any therapy based on induction of apoptosis as a way to cytotoxicity, even when combined with monoclonal antibodies, will result in long-lasting response in CLL patients only if there is functional p53 in tumour cells.[27-30] Such findings resulted in development of new approaches for aberrant p53 CLL populations that rely on *TP53*-independent mechanisms. They include steroid and immunomodulatory agents as well as diverse kinase inhibitors which are thoroughly described in review paper by Shindiapina and colleagues.[31] In summary, because of its significance as a prognostic and therapy responsiveness marker in different subtypes of CLL, *TP53* should be analysed in all CLL patient, both for deletions and mutations at the time of the diagnosis and additionally, during the course of the disease.[32, 33] The gold standard for detection of 17p deletion remains fluorescent *in situ* hybridization (Figure 4), while recommended methods for evaluation of mutations are still debatable.

Sanger sequencing allows evaluation of relatively long fragments with the highest reading sensitivity, but only in a very homogenous sample. In samples where there might be a small fraction of cells forming subclones with certain mutations, such as in CLL subclones with *TP53* mutation, deep next generation sequencing would be more efficient option.[34, 35]

Taken together, the role of p53 in CLL is less important for disease development, but it is highly

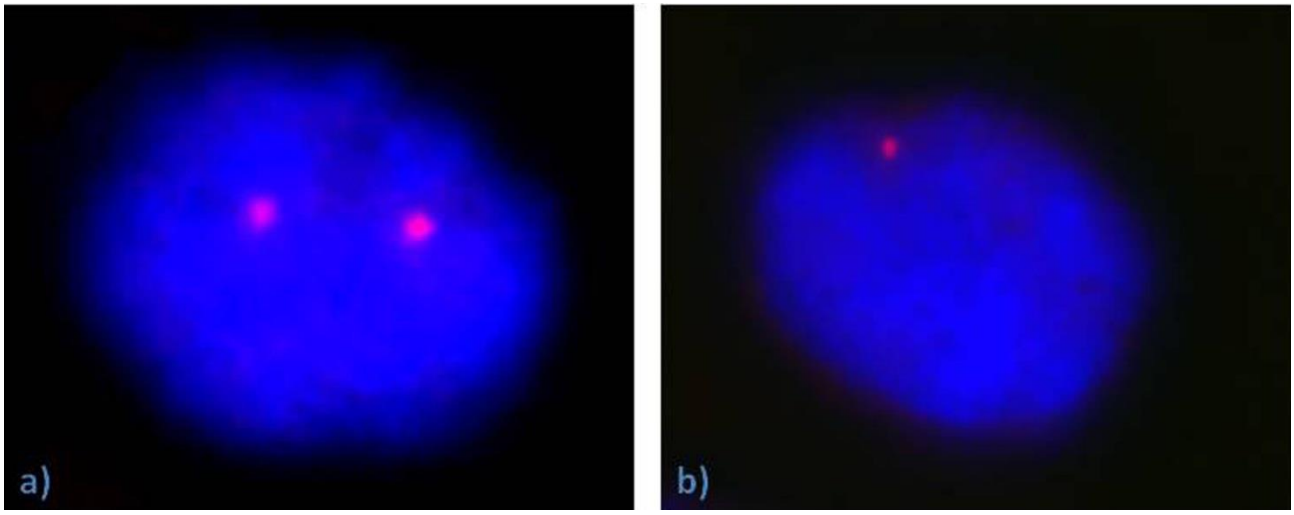

Figure 4. 17p13 deletion detection by fluorescent *in situ* hybridization. a) chronic lymphocytic leukaemia cell without 17p13 deletion, b) chronic lymphocytic leukaemia cell harbouring 17p13 deletion.

informative for appropriate choice of therapy and monitoring course of the disease in treatment-requiring timepoints.

p53 IN MYELODISPLASTIC SYNDROME

Myeloid neoplasms represent dual role of p53 in disease development and progression.[14] Myelodisplastic syndrome (MDS) is a heterogeneous group of diseases marked by ineffective hematopoiesis, cytopenia and dysplasia in one or more hematopoietic cell lines. It is associated with the higher risk for development of acute myeloid leukaemia. MDS mostly develops *de novo*, but high percentage of patients develops therapy-related type.[14] Evaluation of prognosis as well as determination of specific MDS subtypes relies on cytogenetic and molecular profiling of neoplastic cells, based on bone marrow biopsy. In two cytogenetic subtypes p53 has a role in a disease progression. In MDS with 17p loss, there are often *TP53* mutations of the remaining allele correlated with unfavourable clinical behaviour of disease. Those cases are mostly therapy-induced neoplasms.[14] MDS with 5q deletion is recognized as individual entity. Those cases, that are mainly primary neoplasms, have favourable clinical course of the disease when compared with other MDS subtypes. 5q- syndrome is characterized by anaemia and less than 5% of myeloblasts in nucleated bone marrow and megakaryocytes with typical morphological appearance. It is believed that deleted part of the chromosome 5 carries tumour-suppressor genes and that the neoplasm arises due to their loss of activity. One of the genes located on 5q region is *RPS14*, whose loss might contribute to the tumorigenesis due to the loss of ribosomal function. For this entity cell of origin is believed to be hematopoietic stem cell.[14] Even between patients with 5q- syndrome there is considerable heterogeneity: deleted regions are mostly 5q31 and 5q32-33 and are in most cases hemizygous.[36-40] *RPS14* is located on 5q33.1 and coding for component of the ribosomal 40S subunit. In 5q- syndrome it is expressed at 50% level in comparison to MDS without 5q deletion and remaining allele does not harbour additional mutations or any kind of aberrations. When silenced in human hematopoietic stem cell and progenitor cells, its loss results in erythroid block differentiation.[41] Such haploinsufficiency impact on haematopoiesis is caused by activation of wild-type *TP53* which triggers apoptosis of affected cells.[42-46] On the other hand, mutations of *TP53* also contribute to 5q- clinical course.[47] Study conducted on 318 MDS patients showed association of *TP53* mutations and deletion of 5q region both in isolated 5q- cases and cases where 5q- is a part of complex karyotype.[48] In that study patients with MDS having isolated deletion 5q accompanied with a *TP53* mutation showed significantly worse outcome in comparison with patients with 5q- syndrome without *TP53* mutation suggesting the role of aberrant p53 in disease progression.

From still insufficient data about p53 role in myeloid neoplasms it can be observed that it has a dual role: it can be a part of disease development in its wild-type form, but it can also contribute to the progression of otherwise favourable form of the disease when mutated.

VALUE OF P53-STATUS IN COLORECTAL CARCINOMA TREATMENT

p53 is a final step in stepwise development of colorectal carcinoma which suggests its importance for precision therapy development. Colorectal carcinoma (CRC), epithelial tumour of colon or rectum, is one of the most frequent carcinomas in the world. Its aetiology combines environmental and genetic factors. There are many subtypes based on the localization of

the tumour, histology and molecular profiling. They can arise as sporadic forms (75% of cases) or as a consequence of hereditary syndromes/familial susceptibility (25% of cases).[49] In 1990 a stepwise model for CRC was proposed by Fearon and Vogelstein (Figure 5).[50] It describes changes in normal colon tissue that lead to hyperplasia, additional aberrations that drive formation of adenomas and eventually key steps for development of carcinoma. Such progression takes several decades and accumulation of genetic events can be observed during this time and linked to certain steps of tumour development. Although new information is being added to this model, it is still widely accepted and the main step in transition from late adenoma to carcinoma is a mutation/deletion of *TP53* gene. In 2005, Colorectal International Collaborative Study was published.[51] It examined prognostic and predictive value of *TP53* mutation on data of more than 3500 patients from 17 countries. 25 different research groups took part in this study and therefore different screening techniques for *TP53* mutation were used. Analyses were done on exons 4-8 by PCR-SSCP and sequencing, PCR-DGGE and sequencing, only SSCP, only DGGE, or direct DNA sequencing. Results showed that *TP53* mutations are present in 42% of cases with higher percentage in distal colon or rectal tissue, mainly mutation in one allele and different association to survival depending on the site of involvement. The strongest association was shown to be between *TP53* deletions in distal colon tissue and worse survival.[51]
Although there are growing evidences about p53 role in CRC development, the exact role of mutations in tumour cells remains to be elucidated. The strongest

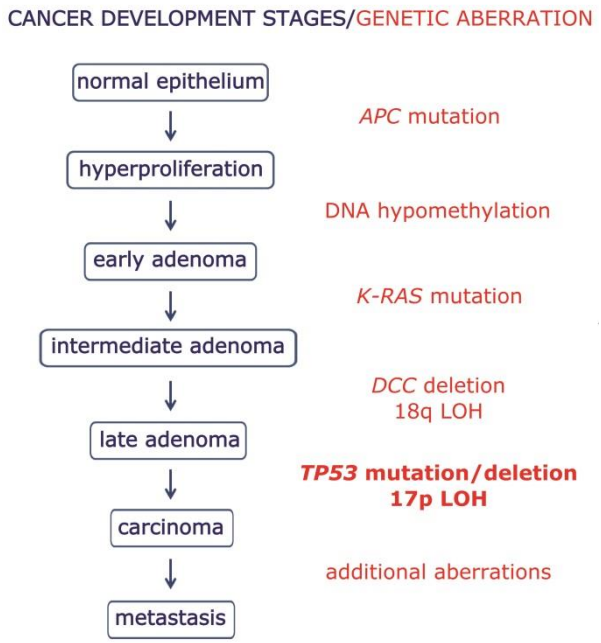

Figure 5. Stages of colon cancer development according to Fearon and Vogelstein (1990).[50]

indications imply its impact on genomic instability. When human colorectal cell line was analysed it was shown that binucleated cells enter mitosis regardless of wild-type p53. Additionally, changes in p53 function prevent centrosome clustering, thus enabling aneuploidy.[52-54] Therefore, mutations of p53 in CRC that induce final step of carcinoma development might be insufficient themselves, but are responsible for additional accumulation of mutations through genomic instability that could be variable depending on the localization of tumour, environmental factors and any other cofounding variable. For that reason new therapies for CRC are being developed that try to reactivate p53 and restore its function.[55-58]

CONCLUSION

TP53 is a tumour-suppressor gene that is thoroughly investigated and linked with various tumour types during tumour development, courses of the diseases, and adequate therapy protocols. In normal cells it has many different roles through different pathways, but it all come to one final function: maintenance of cell genome and integrity. When mutated or even expressed as a wild-type protein in cells that lost their normal phenotype, p53 contributes to the events that are unmistakably associated with tumour mass preservation and tumour evolution inside a host organism. It is recognised as relevant point of tumour progression in neoplasms with different cells of origin, neoplasms at different localizations and as a marker important for promotion of different diseases stages.
Taken together, it is the most investigated and well-known tumour-suppressor that was analysed in many neoplastic models and settings for more than 30 years, but it still not completely understood. At the same time it is represents promising and valuable target for precise cancer treatment protocol design.

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