**TUMOR SUPPRESSOR GENES 2**

Inactivation of tumor suppressor genes plays an important role in the pathogenesis of tumors, for many tumors more important than activation of oncogenes.

In general, the functions of proteins encoded by tumor suppressor genes are:

1) Repression of genes that are essential for the continuation of the cell cycle, which, if not expressed, cause its stoppage;

2) Detection and correction of DNA damage. In a normal cell, as long as these damages exist, it will not continue to divide further. Only when the damage is repaired does the cell cycle resume;

3) Initiation of apoptosis, i.e. programmed cell death in case DNA damage cannot be repaired;

4) Maintaining cell adhesion, blocking the loss of contact inhibition, i.e. inhibition of metastases.

Thus, tumor suppressor genes are very vulnerable to critical DNA damage, and represent a significant physiological barrier to clonal expansion or genetic mutations.

**INACTIVATION OF TUMOR-SUPPRESSOR GENES**

**(The role of tumor suppressor genes in tumor development)**

These genes include the retinoblastoma gene (Rb-1) and p53. The Rb-1 gene inhibits the action of the important transcription factor E2F, and deletion of the Rb gene (seen in hereditary retinoblastoma) relieves E2F suppression. On the other hand, p53 enhances the expression of p21/Cip1, which as a suppressor of cyclin-dependent kinases stops further proliferation.

The importance of Rb-1 and p53 genes in the genesis of tumors has been proven by the identification of mutations of the same genes in people with tumor predisposition syndromes, such as congenital retinoblastoma (Rb-1) and Li Fraumeni multicancer syndrome (p53). Pre-malignant cells have minimal benefit from the inactivation of one copy of the tumor suppressor gene (due to halved effective gene function), or none at all, if the activity of the remaining "wild type" copy of the gene is sufficient for normal function. A significant change in cellular phenotype usually appears only when the function of a tumor suppressor gene is eliminated by two successive inactivating mutations or by a combination of an inactivating mutation and loss of heterozygosity (LOH). The tumor DNA virus, human papillomavirus (HPV), which is the cause of most cervical and perianal tumors, inhibits the action of both mentioned tumor suppressor genes.

Thus, tumor suppressor genes are highly vulnerable to critical DNA damage, and represent a significant physiological barrier to clonal expansion or genetic mutations, and are capable of preventing the growth and metastasis of cells initiated by uncontrolled proliferation mediated by oncogenes.

Loss of function of pRb plays an important role primarily in the carcinogenesis of retinoblastoma. Mutations of the Rb-1 gene, viral oncoproteins, and changes in other proteins involved in the regulation of the pRb pathway play an important role in its inactivation. Rb is also mutated in many other tumors including breast tumor, osteosarcoma, lung tumor, genitourinary tumors, malignant melanoma. The Rb protein normally binds to the E2F transcription factor and inactivates it. Loss of Rb activates E2F, which induces cyclin E gene expression (E2F binds to the cyclin E gene promoter) and synthesis of the Cdk1-cyclin E complex, which further initiates the cell cycle.

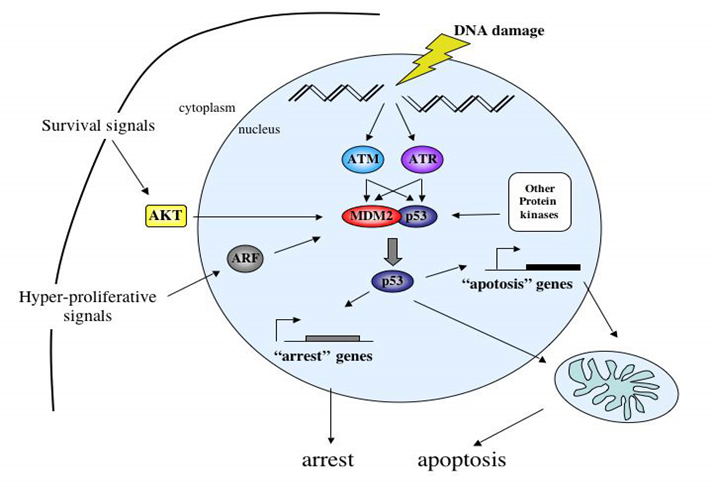
Tumor-suppressor genes lose their function after mutation. The activity of tumor-suppressor genes can be neutralized by interaction with other cellular proteins or with viral oncoproteins. Mutations of the p53 gene directly change the function of the p53 protein, but its function can also be altered under the influence of some oncogenes. Increased oncogene expression sometimes causes sequestration or destabilization of the normal form of the p53 protein, inactivating its tumor-suppressor activity. The critical role of p53 in preventing tumor development is unquestionable, given that p53 gene mutations are present in approximately 50% of human malignancies.

In 1982, an increased frequency of various tumors was discovered in a group of families, including glioblastomas, leukemias, breast, lung, and pancreatic cancers, as well as soft tissue sarcomas. This familial tumor syndrome is called Li-Fraumeni syndrome.

In 1990, 8 years after the onset of this syndrome, researchers discovered that many cases were associated with mutated alleles on chromosome 17p13, specifically p53 alleles. Family members who inherit the mutated p53 allele have a high probability of developing some of the malignant tumors during their lifetime. The time of onset of tumors varies: about 5 years for adrenal cortex cancers, 16 years for sarcomas, 25 years for brain tumors, 37 years for breast cancers and about 50 years for lung cancers.

Today, we know that mutated p53 alleles that are passed on to offspring contain various point mutations.

p53 is a prototype anti-oncogene that plays a significant role in the processes of oncogenesis. The product of the normal p53 gene (wild-type p53) is a protein designated as a transcription factor or DNA-binding protein that exerts its effect both by regulating gene transcription and by interprotein interactions. In normal cells, p53 is generated and degraded continuously, and the half-life of the protein is about 30 minutes. When the genome is damaged, p53 becomes stabilized and the concentration in the cell increases up to ten times as well as the half-life of 24 hours. Its primary function is to monitor checkpoints in the S and M-phase of the cell cycle. It is extremely sensitive to DNA damage, and it is at these checkpoints that it induces a temporary halt in cell division, allowing the enzymes of the DNA repair system to correct this error (damage). In the event that the damage is too great so that it cannot be corrected, it induces either apoptosis, or the irreversible stopping of cell division, the so-called "replicative age" (senescence). In this way, p53 either eliminates or prevents the further division of genetically altered cells (with mutations of oncogenes or tumor-suppressor genes), thereby preventing their accumulation, which pose a risk for tumor development. UV radiation and DNA double-strand breaks by the ATM protein and other DNA-dependent kinases are thought to induce p53 expression. The main target of p53 is p21, an inhibitor of cyclin-dependent kinases. Namely, acting as a transcription factor, it activates the synthesis of the p21 protein. p21 factor can bind to and thus inhibit multiple cyclin-dependent kinases. A high level of this inhibitor blocks the activity of CDK2, and most likely CDK4 and CDK6, stopping the cell cycle in the G1 phase. Then, p53 induces the expression of enzymes of the DNA repair system (*Figure 1*). Not surprisingly, p53 is eliminated or mutated in numerous tumors.



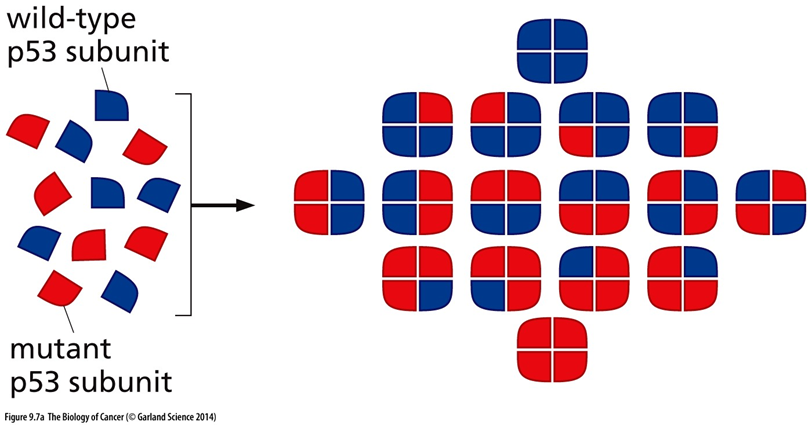
*Figure 1. The primary function of p53 is the control of cell cycle checkpoints. In the presence of damaged DNA, p53 induces a temporary arrest in cell division allowing enzymes of the DNA repair system to repair the damage. If the damage is too great and cannot be corrected then it induces apoptosis and elimination of the genetically altered cells. Mutations in the p53 gene directly alter the function of the p53 protein and cause the accumulation of genetically altered cells that pose a risk for tumor development.*

In response to massive, irreparable damage to genetic material, p53 will initiate apoptosis. p53-induced apoptosis prevents cell survival with the potential to become malignant. The pro-apoptotic effect of p53 is twofold: on the one hand, it inhibits the transcription of Bcl-2 (an anti-apoptotic gene), while on the other hand, it induces the production of the pro-apoptotic Bax protein as well as FAS and other death receptors from the tumor necrosis factor family. The ultimate outcome of the pro-apoptotic action of p53 is the elimination of cells with genetically altered material.

Sequence analyzes of the mutated p53 allele in the genomes of various human tumor cells indicated that the vast majority of mutated p53 alleles carry point mutations in the "reading frames" that create missense codons (by amino acid substitution) and very rarely nonsense codons (which cause premature termination of biosynthesis polypeptide chain). To date, more than 26,000 p53 alleles have been sequenced from human tumor cell genomes; missense mutations were found in 74% of them. Also, deletions of sequences within the "reading frame" of the p53 gene are relatively unusual, as it turns out that tumor cells benefit more from the presence of a slightly altered p53 protein than from its complete absence, which occurs during the formation of null alleles through nonsense mutations or complete deletion of significant parts of the p53 gene. The mutation allows the mutated allele to interfere, or interfere with the ongoing activities of the "wild type" copy of this gene in the cell. Such alleles are therefore called "dominant-interfering" or "dominant-negative" alleles.

Biochemical and structural analysis of the p53 protein revealed that p53 exists in the cell as a homo-tetramer (a set of four identical polypeptide subunits). Consistent with the dominant-negative concept, this tetrameric state suggests a mechanism by which the mutant p53 allele actively interferes with a continuously functional wild-type p53 allele expressed in the same cell.

Suppose that the mutant p53 allele found in human cancer cells encodes a form of the p53 protein that has lost its normal function but participates in tetramer formation. If such a mutated allele coexists with a "wild type" allele in a cell, the p53 tetramers will contain a mixture of the mutated and wild type proteins in different proportions. The presence of only one mutated p53 protein in a tetramer can interfere with the functioning of the tetramer as a whole (*Figure 2*). Therefore, in a cell that is heterozygous for the p53 locus, 15 out of 16 possible p53 tetramer combinations may contain at least one mutated p53 subunit, and thus such tetramers may completely or partially lack the function characteristic of a completely wild-type p53 tetramer.



*Figure 2. Structure and function of p53 in the case of a dominant-negative allele*

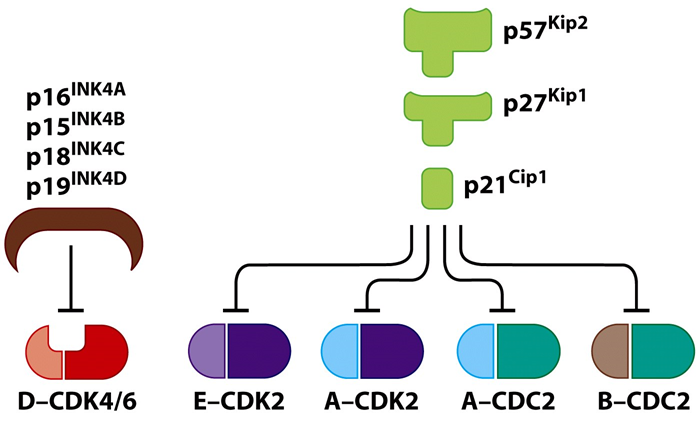
**Short half-life of p53 protein**

Measurement of p53 protein levels indicated that they can vary drastically in different types of cells, and that their rapid increase is more inconspicuous when cells are exposed to some type of physiological stress. This raises the question of the mechanism by which the cell modulates p53 protein levels.

The p53 protein has a half-life of only 20 minutes. Based on this, it was concluded that p53 is a very unstable protein, which is degraded by proteolysis immediately after synthesis. Why would a cell invest a considerable amount of energy and synthetic capacity in the production of a protein molecule, and then immediately destroy it?

The answer lay in the fact that cells need a rapid change in the level of this (as well as other proteins, such as Myc), in order to achieve an adequate response to certain physiological signals.

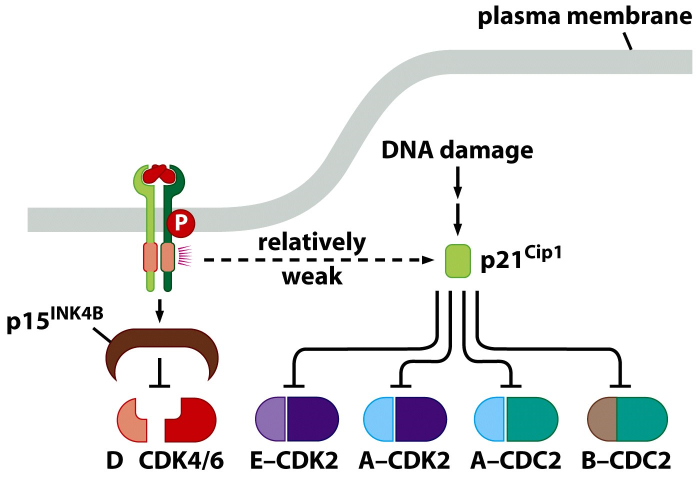
Cyclin-dependent kinase inhibitors (CDK inhibitors) are one of the categories of tumor suppressors. To date, 7 of these proteins are known that can antagonize the activities of the cyclin-CDK complex. CDK inhibitors fall into two categories. The group of 4 mentioned proteins are specific inhibitors of cyclin-dependent kinase 4 (CDK4), also known as INK4 proteins (inhibitors of CDK4). Their targets are CDK4 and CDK6 complexes and have no effect on CDK2. These inhibitors include p16INK4A, p15INK4B, p18INK4C, and p19INK4D. The three remaining inhibitors are non-specific. They include p21, p27 and p57 and inhibit several different cyclin-CDK complexes that are formed during the following phases of the cell cycle (*Figure 3*).



*Figure 3. CDK inhibitor activity*

When TGF-β binds to a receptor on epithelial cells, it initiates multiple signaling pathways that antagonize cell proliferation. These changes include an increase in the level of p15INK4B, which consequently blocks the formation of the cyclin D-CDK4/6 complex (*Figure 4*) and inhibits already existing complexes. Without active cyclin D-CDK4/6 complexes, the cell is unable to "progress" from the G1 phase of the cell cycle. When the cell "exits" from the G1 phase, the activity of the cyclin D-CDK4/6 complex becomes unnecessary. This may explain why TGF-β inhibits cell growth in the early G1 phase and loses most (almost all) of its inhibitory properties when the cell passes the G1 phase.

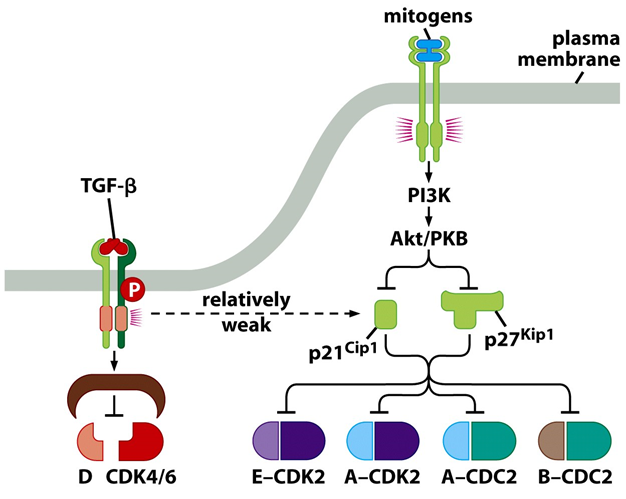
TGF-β also induces the activity of p21Cip1, a broad-acting CDK inhibitor, although much weaker. A significant increase in the activity of the p21Cip1 inhibitor occurs as a response of the cell to various damages (eg DNA damage; *Figure 4*). When present in a significant amount in the cell, p21Cip1 acts in all phases of the cell cycle and stops further "progress" of the cell.



*Figure 4. Mechanisms of TGF-β cell cycle control*

While DNA damage and, to a much lesser extent, TGF-β can increase the level of p21Cip1 in the cell and thus arrest the cell cycle, mitogens have the opposite effect. They block the activity of CDK inhibitors and accelerate the cell cycle. One of the mechanisms of mitogen action is through the phosphatidyl-inositol 3-kinase pathway (PI3K; *Figure 5*).

Akt/PKB, a kinase activated downstream of the PI3K signaling pathway phosphorylates nuclear-localized p21Cip1 molecules and causes them to exit the nucleus into the cytoplasm, where they cannot inhibit cyclin-CDK complexes. In a similar way, Akt/PKB inhibits the function of the p27Kip1 molecule as well. In aggressive tumors, Akt/PKB kinase is very active, and inhibitors of the cyclin-CDK complex are not found in the nucleus, but in the cytoplasm.



*Figure 5. Cell cycle control by extracellular signals*

**The role of anti-oncogenes in the elimination of DNA damage**

In addition to arresting the cell cycle, the protein products of anti-oncogenes also coordinate the detection and correction of genetic errors. The signaling network that controls these mechanisms is driven by the main DNA damage sensors ataxia-telangiectasia mutated protein kinase (ATM) and AT and Rad3-related protein kinase (ATR). ATM generally recognizes both DNA strand breaks, while ATR recognizes replication fork damage and large DNA damage. When they recognize DNA damage, both proteins phosphorylate subsequent signaling molecules. The main signal transmitters are CHK2 (activated by ATM) and CHK1 (activated by ATR). p53 is a major substrate of ATM/CHK2 and ATR/CHK1 phosphorylation. Activation of p53 is the main mechanism by which the DNA damage regulatory system responds to detected damage. DNA damage during replication, unrelated to replication, hypoxia, telomere dysfunction are just some of the activators of p53, via the aforementioned signaling network. One of the main effects of p53 is the activation of p21 and subsequent inhibition of cyclin E(A)/ CDK 2 and cell cycle arrest.

**Immortalization and oncogenesis**

"Death occurs because exhausted tissue cannot be endlessly renewed."

and because the number of cell divisions of an individual cell is finite."

August Weissmann, biologist 1881.

During tumorigenesis, cells break through a barrier that limits their replicative potential. They acquire the ability to divide a large number of times, so that they go through a multi-stage process very successfully.

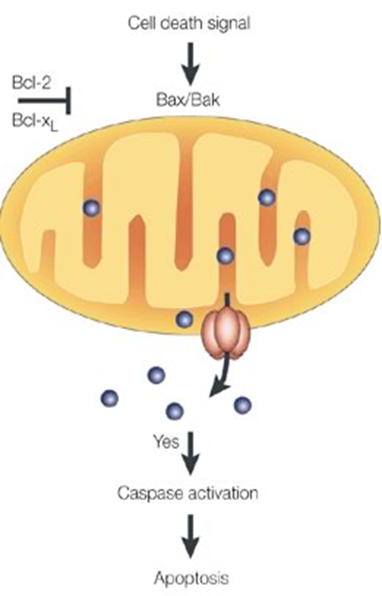
In 1960, Leonard Hayflick showed for the first time that fibroblasts, grown in culture, have a limited replicative potential (50-100 divisions). When cells "exhaust" their proliferative capacity, they enter a stage called replicative age. Limiting cell division to a finite number can represent a mechanism of protection against the growth and development of malignancy, and is a consequence of telomere shortening. Telomere shortening is a unique mechanism by which the proliferative potential of the cell is limited. Cells that lack this process continue to proliferate. Numerous studies indicate that it is an important and evolutionarily conserved tumor-suppressor mechanism and represents a natural barrier for immortalization and malignant transformation of cells. Telomeres are specialized nucleoprotein complexes located at the ends of chromosomes. With their topographical position, they "cover" the ends of chromosomes. Thus, they prevent the recognition and repair of breaks in the DNA double helix by repair mechanisms. DNA polymerase that functions during the S phase of the cell cycle requires an RNA primer to initiate replication, resulting in incomplete replication of telomere DNA during each cell division. With each cell division, telomeres shorten (by 25-200bp). This problem can be solved by telomerases - enzymes that synthesize telomeres, but under physiological conditions the level and activity of these enzymes is not sufficient to maintain the length of telomeres. Shortened telomeres cannot adequately protect chromosome ends from repair mechanisms, which results in the accumulation of cdk inhibitors and cell cycle arrest. A fundamental role in this control mechanism is played by the p53 and pRb pathways, which are critical for its initiation and maintenance. Telomere shortening represents a kind of mitotic clock that limits the number of divisions and the life span of the cell.

In early embryogenesis, cells have unlimited replicative capacity. Differentiated cells (neurons, mammary epithelial cells, skin fibroblasts) have a limited capacity to divide.

Increased telomerase activity is found in most primary tumors. Telomerases are potential targets in anti-tumor therapy. Also, mutations of the tumor suppressors p53 and pRb, along with the p16INK4a mutation, are sufficient to block cellular senescence, thereby removing the barrier to tumor progression.

**Inhibition of apoptosis**

An essential feature of malignant transformation is the blocking of apoptosis, which prolongs the life of the cell and makes it immortal. Normal cells have control mechanisms that induce apoptosis under different conditions: when the number of mutations reaches a critical level, then under the influence of cytokines (TNF, IL-3) and after DNA damage. The malignant cell "keeps the mechanisms of apoptosis under control", that is, it blocks apoptosis through the anti-apoptotic proteins of the Bcl family. The first identified member of this family is the Bcl-2 gene (B Cell Lymphoma gene-2). It encodes a cytoplasmic protein that, together with its homolog Bcl-x, inhibits apoptosis by blocking the function of pro-apoptotic proteins and preserving the integrity of the mitochondrial membrane (*Figure 6*). Bcl-2 inhibits apoptosis when it is overexpressed, or expressed in an inadequate tissue. Apoptosis inhibition mechanisms play an important role in tumor development. Bcl-2 gene expression disorder causes increased levels of mRNA and protein that accumulate primarily in hematological malignancies (acute myeloid leukemia, acute and chronic lymphocytic leukemia), but also in solid tumors such as lung cancer. Overexpression of Bcl-2 prolongs cell life and allows the accumulation of mutations and rearrangement of oncogenes, such as c-myc and Ras. It is believed that the cooperative action of Bcl-2 and c-myc induces malignant transformation, indicating that the development of malignancy requires the activation of more than one oncogene. Additionally, the results of experimental research show that transfection of fibroblasts with individual plasmids programmed to express proteins involved in either cell growth or immortalization does not result in malignant transformation. However, co-transfection of two plasmids (one for induction of proliferation, the other for immortalization) results in transformation.



*Figure 6. Inhibition of apoptosis*

**AUTOPHAGY AND ONCOGENESIS**

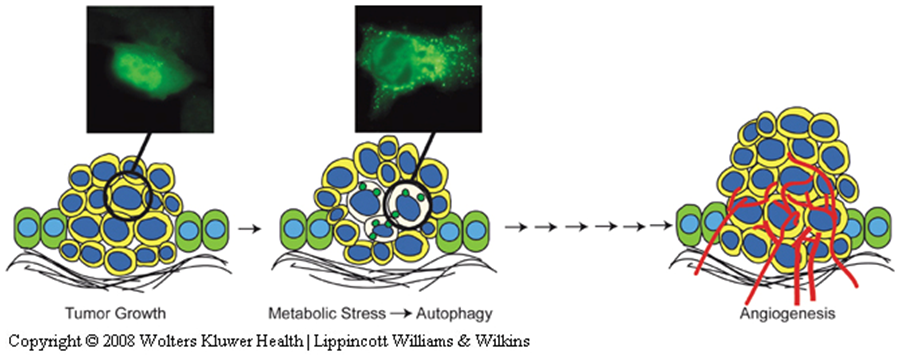
Autophagy or autophagocytosis is an evolutionarily conserved catabolic process of degradation of cellular components (proteins and cellular organelles) by lysosomal machinery. Autophagy enables the short-term survival of nutrient-deprived tumor cells by recycling their own nutrients/building blocks. Tumor cells "use" autophagy to survive metabolic stress. Epithelial tumor cells rapidly proliferate multilayered. Insufficient blood supply induces metabolic stress in the parts of the tumor furthest from nutrients and oxygen, inside the tumor. Tumor cells with defective apoptosis (inhibition of apoptosis), in regions of metabolic stress, can survive by autophagy.

Autophagy is believed to play an important role in tumor development. The intensity of proteolysis in autophagy in cancer cells is lower than in normal cells. This suggests a link between carcinogenesis and reduced levels of autophagy.

In the early stages of a tumor, autophagy acts as a tumor suppressor. The early stages of tumor development require a higher level of protein synthesis from the cancer cell for tumor growth. Therefore, inhibition of autophagy can maintain continuous tumor growth. In addition, autophagy is thought to reduce mutation rates and suppress oncogenesis by eliminating damaged organelles that produce genotoxic factors such as free radicals.

Although autophagy is suppressed in the early stages of tumorigenesis, it appears to be increased during the later stage of tumor progression as a protective mechanism. As the tumor grows, the cancer cells in the periphery continue to multiply. On the other hand, cancer cells located in the central areas of the tumor, which are poorly vascularized, do not have enough nutrients, so the induction of autophagy allows them to survive in conditions with low values of oxygen and nutrients.

Also, autophagy induces local neoangiogenesis (*Figure 7*), through intensifying the proliferation and migration of endothelial cells, which facilitates tumor growth and development.



*Figure 7. Autophagy and tumor genesis and progression*

However, autophagy, as programmed cell death type II, also plays a role in the elimination of tumor cells. The gene encoding Beclin-1, a major autophagy-triggering protein, is often down-expressed in many tumor types, and deletion of this gene in mice significantly increases tumor incidence.