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Epigenetic Alteration of Receptor Tyrosine Kinases in Cancer

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1. Introduction

In the process of cellular carcinogenesis, genetic and epigenetic mechanisms contribute to abnormal expression of genes. Conrad Waddington introduced the term “epigenetics” in 1942 (Goldberg, Allis et al. 2007) to describe heritable changes in gene expression that have no connection with changes in DNA sequence (Yoo and Jones 2006; Goldberg, Allis et al. 2007). The molecular basis of epigenetics is complex and involves DNA methylation, histone modifications, chromatin remodeling and microRNAs (Esteller 2006).

The most studied epigenetic mechanism is DNA methylation (Urduingio, Sanchez-Mut et al. 2009) defined as the covalent addition of a methyl group at the 5'-position of cytosines within CpG dinucleotides, a process usually related to gene silencing in eukaryotes (Fig. 1A). In the human genome, the CpG dinucleotides are generally gathered in regions called CpG islands, preferentially located in promoter regions and usually not containing 5-methylcytosines (Bird 2002; Esteller 2008). Between 60% and 90% of all CpGs are methylated in mammals, to prevent chromosomal instability (Ehrlich, Gama-Sosa et al. 1982).

While DNA methylation of CpG islands is required by many physiological processes during normal development, aberrant DNA methylation can cause several pathologies, including tumor formation. (Bestor 2000; Reik, Dean et al. 2001). In the process of cellular carcinogenesis, several epigenetic alterations contribute to the abnormal expression of genes. For example, aberrant DNA methylation patterns (hypermethylation and hypomethylation) have been described in a large number of human malignancies (Fig. 1B). Hypermethylation is one of the major epigenetic modifications and typically occurs at CpG islands in the promoter region, leading to gene inactivation (Fig. 1B). Gene promoters that are aberrantly methylated during tumor development offer valuable insight on the biological pathways

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that are commonly interrupted during tumorigenesis (Klarmann, Decker et al. 2008; Suzuki, Toyota et al. 2008) . Gene promoter hypermethylation is related to the inhibition of cancer-related genes such as tumor suppressor genes and DNA mismatch repair genes (Feinberg and Tycko 2004; Yoo and Jones 2006) (Fig. 1B). Several key tumor suppressor genes have been found to exhibit promoter hypermethylation more often than genetic disruption (Chan, Glockner et al. 2008).

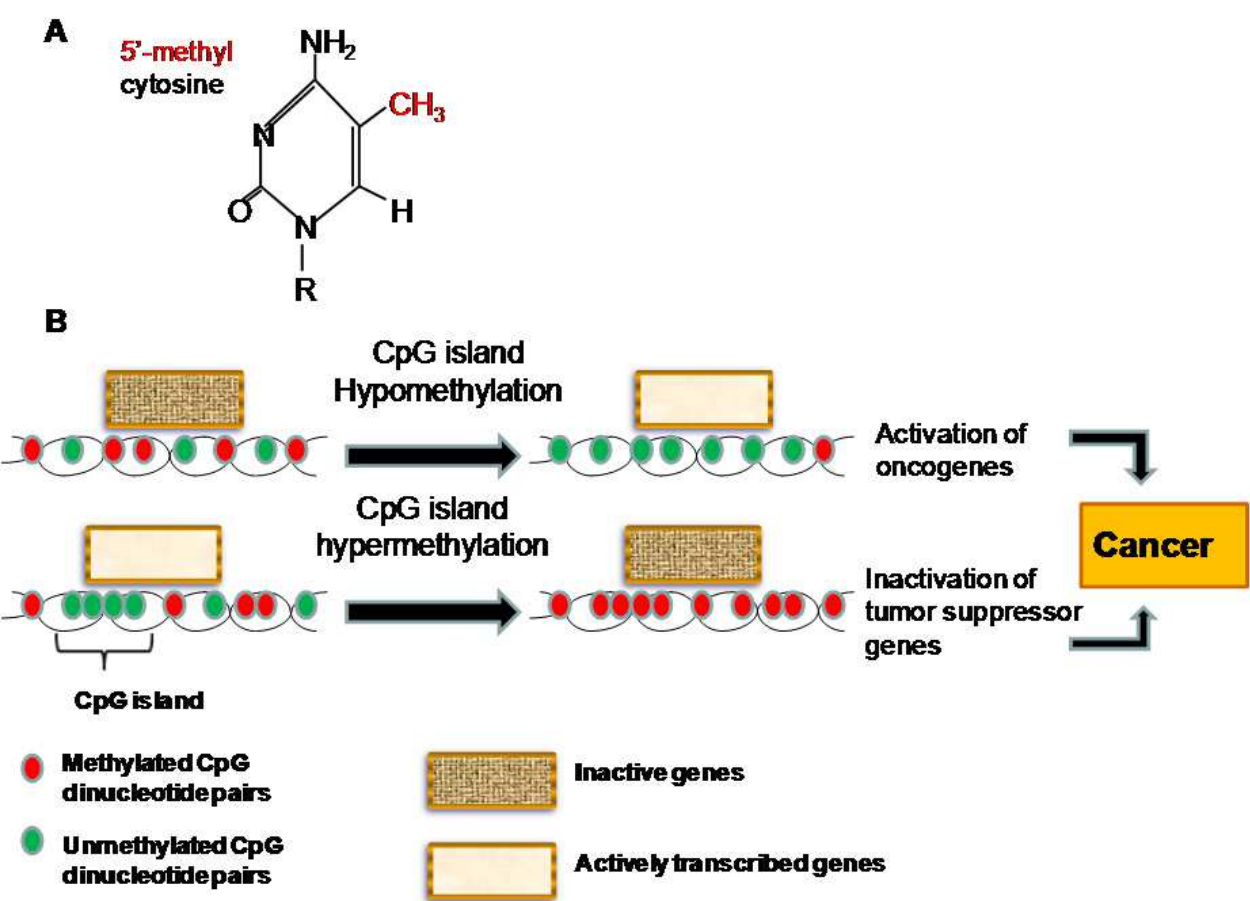


Fig. 1. Aberrant DNA methylation is involved in the development of cancer.

DNA methylation is a process of covalent addition of a methyl group at the 5'-position of cytosines (A) within CpG dinucleotides. DNA hypermethylation is commonly interrupted during tumorigenesis and is related to the inhibition of tumor suppressor genes and DNA mismatch repair genes while DNA hypomethylation appears to be involved in tumor cell development by activation of oncogenes (B).

Global hypomethylation has also been implicated in the development and progression of cancer, through different mechanisms (Yoo and Jones 2006). DNA hypomethylation, which is the first epigenetic event identified in cancer cell (Feinberg and Tycko 2004), appears to be involved in tumor cell development by activation of oncogenes, generation of chromosomal instability and loss of imprinting (Feinberg and Tycko 2004) (Fig. 1B). The low level of DNA methylation in tumors can increase the expression of several oncoproteins involved in cell growth and survival, apoptosis and cell cycle regulation (Jun, Woolfenden et al. 2009).

Among all oncogenes, receptor tyrosine kinases (RTKs) play crucial roles in the control of cancer cell growth and differentiation. RTKs are transmembrane proteins with intrinsic intracellular tyrosine kinase activity (Fig. 2).

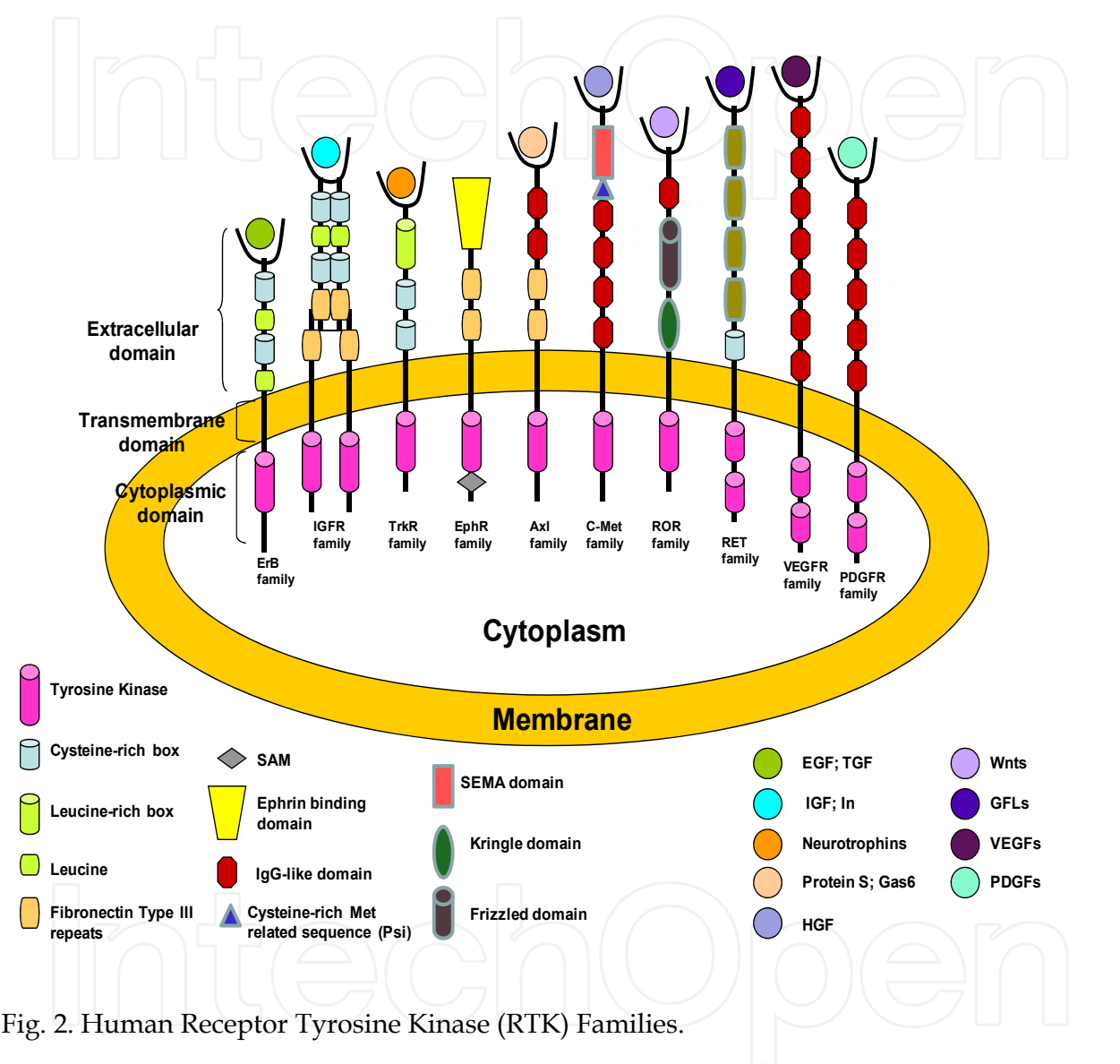


Fig. 2. Human Receptor Tyrosine Kinase (RTK) Families.

RTKs are membrane proteins, composed by an extracellular ligand-binding domain and an intracellular catalytic domain. Based on the structure of their intracellular domain and the nature of their specific ligand, RTKs are grouped in distinct families: ErbB (epidermal growth factor receptor) family, IGF-1R (Insulin-like Growth Factor 1 Receptor) family; TRK (tropomyosin receptor kinase) family, EphR (erythroprotein-producing hepatoma amplified sequence receptor) family, AXL (AXL receptor) family, c-Met (hepatocyte growth factor receptor or Scatter factor receptor) family, ROR (retinoid-related orphan receptors) family, RET (rearranged during transformation) family, VEGFR, (vascular endothelial growth factor receptor) family and PDGFR (platelet-derived growth factor receptor).

RTKs overexpression or overactivation has been described in almost all tumor types. The human genome is reported to contain 58 genes encoding receptor protein kinase, grouped into 20 classes, or subfamilies, based upon their kinase domain sequence (Robinson, Wu et al. 2000; Lemmon and Schlessinger 2010). Many RTK genes contain a typical CpG island and alterations in RTK promoter methylation have been linked to cancer development and progression (Datta, Kutay et al. 2008). Furthermore, emerging data suggests that acquired resistance to conventional cancer therapy results from progressive accumulation of RTK epigenetic modifications.

We review herein the epigenetic alterations of ten RTK families (Fig. 1), discuss their role in tumor development and implications for the response of cancer cells to conventional therapy.

2. ErbB family

ErbB protein family, or epidermal growth factor receptor (EGFR) family, consists of four structurally related receptors, with a well described tyrosine kinase activity: ErbB-1, also called epidermal growth factor receptor (EGFR), ErbB-2, named HER2 in humans and neu in rodents, ErbB-3, also named HER3 and ErbB-4 or HER4 (Bublil and Yarden 2007). ErbB-1 and ErbB-4 bind different polypeptide extracellular ligands including the epidermal growth factor (EGF), transforming growth factor α (TGF α), amphiregulin, betacellulin, epiregulin, heparin binding EGF, epigen, and neuregulins 1-4, which share a conserved epidermal growth factor (EGF) domain, giving rise to a diverse signaling network (Citri and Yarden 2006). ErbB2 is an “orphan” receptor and ErbB3 lacks the tyrosine kinase activity, hence, both signal through ErbB family heterodimers (Alimandi, Romano et al. 1995).

ErbB receptors/ligands are involved in many fundamental processes during organogenesis and adulthood: cell growth, differentiation, proliferation, apoptosis, motility, invasion, repair, survival and cell-cell interaction (Yarden and Sliwkowski 2001). In tumors, there is abnormal signalling via the ErbB pathway, mostly as a result of receptor overexpression or constitutive activation (Yarden and Sliwkowski 2001).

The *egfr* proto-oncogene localizes on chromosome 7p12, and the promoter region was described as a CG rich sequence, which lacks a TATA box and displays a large CpG island that extends into exon 1 (Kageyama, Merlino et al. 1988). EGFR expression is primarily regulated at the mRNA levels (Xu, Richert et al. 1984; Merlino, Ishii et al. 1985) and many human malignancies with epithelial origin including brain, head and neck squamous cell carcinoma (HNSCC), breast, esophagus, gastric, colon and lung cancers are characterized by EGFR overexpression. In glioma, lung, ovarian and breast cancers, mutations in the *egfr* gene are responsible for the receptor overactivity (Moscatello, Holgado-Madruga et al. 1995). Amplifications at *egfr* locus were also described in gliomas (Li, Chang et al. 2003), colorectal cancers, and less frequently in breast cancer (Al-Kuraya, Schraml et al. 2004; Ooi, Takehana et al. 2004). Genetic polymorphism of *egfr* in the intron 1 region, involving CA single sequence repeats (SSR), constitutes another mechanism that may influence *egfr* gene transcription (Gebhardt, Zanker et al. 1999). More recently it was shown that DNA methylation may also be responsible for the aberrant transcription of *egfr* in neoplastic cells (Kulis and Esteller 2010). Petrangeli et al., attempted for the first time to reveal the implication of DNA methylation in the control of EGFR function in breast cancer patients.

However, a methylation profile comparison of *egfr* promoter in tumoral versus perineoplastic tissues could not identify any differences in *egfr* gene methylation (Petrangeli, Lubrano et al. 1995). In colorectal cancer, Montero et al., identified *egfr* methylation only in the adjacent normal colon tissue but not in the corresponding tumor tissue analyzed (Montero, Diaz-Montero et al. 2006). In contrast with these results, Scartozzi et al., identified *egfr* promoter methylation in 39% of the colorectal tumors samples (Scartozzi, Bearzi et al. 2011). Furthermore, in a subsequent study of a larger cohort, the same authors showed that 58% of patients treated with monoclonal antibodies targeting EGFR had *egfr*, monoallelic or biallelic methylated (Scartozzi, Bearzi et al. 2011) and revealed a direct correlation between *egfr* methylation status and clinical outcome. However, no correlation between the methylated *egfr* and absence of protein expression was evident, as EGFR protein was detected in samples displaying a methylated *egfr*.

Different methylation density in the *egfr* promoter was also identified in three breast cancer cell lines (MB435, CAMA1, and MB453), in SAOS-2 human osteosarcoma cell line, SF-539 glioma cell line, and in two hematopoietic cell lines Raji and Raji Dac where a clear association between the methylated *egfr* and the loss of protein expression was evident (Montero, Diaz-Montero et al. 2006). Furthermore, a low density of methylated cytosines within the *egfr* promoter was also identified in 20% of primary breast, 11% of lung and 35% of head and neck tumors. It is therefore apparent that *egfr* promoter methylation is one potential mechanism responsible for the transcriptional control of *egfr* oncogene in cancer.

The second member of the EGF receptor family, Her-2/neu proto-oncogene, located on human chromosome 17q21 contains, similarly to EGFR, a large CpG island within the gene promoter region. Even though the regulatory region of the gene displays more than 10 potential sites of methylation, no aberrant methylation of *her2* could be detected in different tumor samples, including breast, lung, colon, head and neck and cell lines (Montero, Diaz-Montero et al. 2006). Nonetheless, in epithelial ovarian cancer, the expression of DNA demethylase (dMTase) correlates with poor methylation of *her2*, suggesting that dMTase enzyme is involved in Her2 promoter demethylation (Hattori, Sakamoto et al. 2001). In addition, overexpression of the rat neu oncogene in mammary tumors of MMTV/c-neu transgenic mice induced demethylation of the MMTV promoter (Zhou, Chen et al. 2001). In the same mouse model Kmiecik et al., later showed that Interferon γ (IFN- γ) mediates epigenetic changes in neu oncogene, resulting in neu antigen loss and tumor escape (Kmiecik, Knutson et al. 2007).

Only a paucity of data describing the transcriptional control by any epigenetic mechanisms of Her 3 and Her 4 currently exists. DNA methylation of a CpG island in the promoter region seems to be involved in *her4* transcriptional suppression in breast cancer cell lines and primary breast carcinomas. Moreover, Das, P.M., et al., demonstrated a direct correlation between the *her4* methylation status and HER4 protein expression and proposed *her4* promoter methylation as a negative prognostic indicator at least in breast cancer (Das, Thor et al. 2010).

Identification of DNA methylation as a regulatory mechanism and a prognostic marker for three out of four EGF receptors provides promising new alternatives to targeting ERB signaling with better selectivity, safety and efficacy.

3. IGF receptor family

The insulin-like growth factor (IGF) family is composed of two ligands – (IGF-1 and IGF-2), two receptors – (IGF-1R and IGF-2R), six high-affinity IGF-binding proteins – (IGFBP 1-6) and several associated IGFBP degrading enzymes (proteases). The IGF family has a critical role in the development and maintenance of normal tissue homeostasis and it appears to be involved a number of pathological states, including cancer.

Insulin-like growth factor type-1 receptor (IGF-1R), the main receptor of IGF family, is a transmembrane heterotetramer, linked to the PI3K/Akt and MAPK signal transduction pathways. Approximately 75% of the IGF-1R promoter region consists of cytosine and guanine. The IGF-1R promoter is also TATA-less and CCAAT-less and contains several potential binding sites for Sp1, ETF and AP-2 nuclear transcription factors (Werner, Stannard et al. 1990). IGF-1R is overexpressed in several types of cancer and their involvement in cancer cells' response to treatment has also been reported (Kanter-Lewensohn, Dricu et al. 2000; Cosaceanu, Budiu et al. 2007). In the human IGF-1R promoter region, bioinformatic analysis revealed the presence of multiple CpG dinucleotides (Schayek, Bentov et al. 2010). The presence of CpG islands in the promoter region of IGF-1R gene supports the theory that an altered methylation status of the IGF-1R promoter may be responsible for the IGF-1R oncogene overexpression in various cancers. In 2010, we described for the first time the partial methylation of IGF-1R promoter in three subtypes of non-small cell lung cancer (NSCLC): large cell lung cancer, squamous cell carcinoma and adenocarcinoma. We found the same level of IGF-1R promoter methylation in all NSCLC subtypes and no correlation between the gene methylation level and receptor protein expression (Ola 2010).

While several studies suggest IGF-1R overexpression as a key in prostate cancer initiation and progression (Hellawell, Turner et al. 2002; Turney, Turner et al. 2011), Chott et al. demonstrate that loss of IGF-1R may contribute to prostate cancer progression (Chott, Sun et al. 1999). In line with these findings, Schayek et al., 2010, showed that progression towards metastatic stages in prostate cancer is correlated with a dramatic reduction in both total IGF-1R protein levels and basal phospho-IGF-1R values, which reflects a decrease in IGF-1R activation (Schayek, Bentov et al. 2010). Furthermore, studies on the IGF-1R methylation of six prostate cancer cell lines showed that the IGF-1R promoter is unmethylated in all examined cell lines, suggesting that the gene silencing in metastatic prostate cancer is probably not caused by direct gene promoter methylation (Schayek, Bentov et al. 2010). However, our studies from two glioblastoma lines suggest that IGF-1R is, at least in this setting, partially methylated (Ola 2010).

Many promising agents that inhibit the key enzymes involved in establishing and maintaining the epigenetic changes have been identified and tested in various types of pathologies. One of those agents is S-Adenosylmethionine (SAM), a methyl donor agent. Several studies have reported DNA hyper-methylation, after SAM exposure (Fuso, Cavallaro et al. 2001; Detich, Hamm et al. 2003). In contrast, evidence from our studies on S-Adenosylmethionine-induced cytotoxicity in both glioblastoma and non-small cell lung cancer cell lines demonstrates that SAM does not affect the IGF-1R methylation status (Ola 2010; Ola 2010). While IGF1R epigenetics seems important, the exact mechanisms underlying IGF-1R overexpression in cancer are far from being understood.

Insulin-like growth factor type-2 receptor (IGF-2R, also known as the cation-independent mannose 6-phosphate receptor), the second cell-surface receptor of the IGF family, is a 275 ± 300 kDa glycoprotein, comprised of an extracytoplasmic domain made up of 15 contiguous cysteine-rich repeats, a single transmembrane-spanning domain and a carboxyl-terminal cytoplasmic domain (Ghosh, Dahms et al. 2003). The receptor does not appear to have any protein kinase activity but the carboxyl-terminal cytoplasmic domain has been shown to facilitate endocytosis (Probst, Puxbaum et al. 2009) and intracellular sorting of lysosomal enzymes (Munier-Lehmann, Mauxion et al. 1996). IGF-2R is a maternally expressed gene regulated by epigenetic modifications and one of the classical examples of tissue-specific and species-dependent imprinted genes. There are several studies demonstrating that IGF2 ligand is also an imprinted gene and it has been proved that loss of imprinting causes proliferation of transformed cells in Wilm's tumors, by elevating the level of available IGF2 ligand (Steenman, Rainier et al. 1994; Vu 1996). Both IGF2 and IGF2R genes possess a CG-rich and TATA-less promoter (Szentirmai, Yang et al. 2003). In mice, methylation of region 2, a region rich in cytosine-guanine doublets in the second intron of IGF-2R, represents the imprinting signal that maintains expression of the maternal allele (Stoger, Kubicka et al. 1993). Wutz and his colleagues tested the role of region 2 and the influence of chromosome location on IGF-2R imprinting, using mouse YAC-T1/P and YAC-T1/PAR2 transgenes (Wutz, Smrzka et al. 1997). Their results show that deletion of region 2 of the IGF-2R gene is associated with loss of imprinting and restoration of biallelic IGF2R expression, demonstrating the primary role for the region 2 and the negligible role for chromosomal location in IGF2R imprinting (Wutz, Smrzka et al. 1997).

Increasing evidence from genetic studies currently implicate IGF-2R as a tumour suppressor in a variety of malignancies. Loss of heterozygosity (LOH) at the gene locus on $6q26 \pm 27$ has been reported for a number of tumour types, including breast (Chappell, Walsh et al. 1997), liver (Oka, Waterland et al. 2002), lung (Kong, Anscher et al. 2000) and head and neck (Jamieson 2003) cancers. Further investigations of the involvement of both IGF-1R and IGF-2R and their interaction with the ligands are required to fully understand the role of these complex molecules in cancer.

Unlike genetic changes, epigenetic events do not alter the DNA code and are potentially reversible. Therefore, reactivation of epigenetically silenced genes like IGF2-R could provide attractive therapeutic opportunities.

4. C-MET receptor family

The *c-met* oncogene is a tyrosine kinase receptor binding to the hepatocyte growth factor (HGF) also known as the scatter factor (SF). Signals through c-Met are required for normal mammalian development and fundamental processes like cell migration, differentiation, proliferation, cell growth, branching and angiogenesis (Birchmeier, Birchmeier et al. 2003). Although HGF is the only known ligand for C-Met, glial cell derived neurotrophic factor (GDNF) also seems to be able to activate C-Met, albeit indirectly (Popsueva, Poteryaev et al. 2003).

C-met gene has been mapped to chromosome 7q31 and its regulatory region is GC-rich. It contains no TATA box, and displays a CpG island with an increased frequency of CpG, suggesting that an aberrant transcriptional regulation may play a critical role in oncogene activation (Seol and Zarnegar 1998).

Many studies to date provide evidence that dysregulation of c-Met activity is a key event in the initiation and progression of carcinogenesis (Maulik, Shrikhande et al. 2002). Mutations in *c-met* were identified in papillary renal carcinoma, gastric and liver cancer, small and non-small cell lung cancers, and head and neck squamous cell carcinomas (Peruzzi and Bottaro 2006). Furthermore, amplification of *c-met* in gastric, colorectal and lung carcinoma seems to inversely correlate with survival (Kuniyasu, Yasui et al. 1992; Zeng, Weiser et al. 2008; Lee, Seo et al. 2011).

The possibility that an altered methylation of *c-met* promoter induces overexpression of C-MET protein was, for the first time, addressed in papillary carcinoma although no changes in c-Met promoter methylation could be detected in tumour tissue compared to normal tissue (Scarpino, Di Napoli et al. 2004). Evidence from Morozov et al demonstrated that the death-domain associated protein (Daxx) (a component of a multiprotein repression complex), preferentially binds to the *c-met* promoter together with Histone deacetylase 2 (HDAC2), leading to *c-met* transcriptional repression (Morozov, Massoll et al. 2008). However, DNA methylation seems not to be involved in the Daxx-mediated repression of the *c-met* promoter (Morozov, Massoll et al. 2008). Further studies are needed in order to elucidate the possible implication of c-met promoter methylation in carcinogenesis.

Despite the transcriptional repression of *c-met* proto-oncogene by chromatin modifications, direct evidence of DNA methylation as a responsible mechanism for *c-met* promoter function is missing and needs to be further investigated.

5. Trk receptor family

Tropomyosin-receptor-kinase (Trk) receptors belong to a family of tyrosine kinases that control synaptic strength and plasticity in the mammalian nervous system (Huang and Reichardt 2003). Trk receptors bind neurotrophins and trigger downstream activation of several signaling cascades that affect normal physiological processes, like neuronal survival and differentiation (Segal 2003).

TrkA, TrkB, and TrkC are the three most common types of trk receptors. Each of them has different binding affinity to certain types of neurotrophins. The receptors act through different intracellular pathways and the differences in their signaling can generate a diversity of biological responses (Segal 2003). The Trk oncogene was initially identified in a colon carcinoma and its identification led to the discovery of the first member, TrkA (Huang and Reichardt 2003). The oncogene was produced by a mutation in chromosome 1 that resulted in the fusion of the first seven exons of tropomyosin to the transmembrane and cytoplasmic domains of the then-unknown TrkA receptor (Martin-Zanca, Hughes et al. 1986).

Accumulating evidence now suggests that TrkA, TrkB and TrkC play an important role in the malignant behavior of cancer cells, like increased metastasis, proliferation and survival (Sclabas, Fujioka et al. 2005; Jin, Kim et al. 2010). Studies on different tumors show that TrkC seems to be highly expressed in neuroblastoma, medulloblastoma, (Segal, Goumnerova et al. 1994; Yamashiro, Liu et al. 1997; Grotzer, Janss et al. 2000) and breast cancer (Bardelli, Parsons et al. 2003; Wood, Calhoun et al. 2006). Furthermore, overexpression of TrkA was reported in papillary thyroid and colon carcinoma (Nakagawara 2001), and of TrkB in malignant keratinocytes (Slominski and Wortsman 2000) and pancreatic cancer (Sclabas,

Fujioka et al. 2005). Recent evidence from Jiin et al show that while Trks might be important for liver cancer metastasis and are highly expressed during the course of tumor progression, no aberrant promoter methylation could be detected. In contrast, low or undetectable expression level of Trk receptors in normal liver cell lines demonstrated a dramatically increased pattern of methylation (Jin, Lee et al. 2011).

Given their high level of expression in cancer and their roles in metastasis, current studies now attempt to decipher their functions as oncogenic tyrosine kinases during malignant transformation, and to define their potential as attractive targets for therapeutic intervention (Shawver, Slamon et al. 2002).

6. Eph receptor family

The erythropoietin-producing hepatoma amplified sequence (Eph) receptor tyrosine kinase family is the largest family of tyrosine kinases, comprising at least 14 Eph receptors and 8 ligands (Manning, Whyte et al. 2002). Based upon sequence similarities in their extracellular domains and their ability to bind to ligands, Eph receptors are divided into two groups, EphA and EphB. The EphA members (EphA1-A8 and A10) bind to the glycosyl-phosphatidyl-inositol (GPI) - linked ligands EFNA1-5, whereas EphB1-B4 and B6 belonging to the EphB receptor family binds the transmembrane ligands EFNB1-3 (Pasquale 2004). Upon ephrin binding, Eph receptors are clustered, phosphorylated and kinase activated (Bruckner and Klein 1998). Different pathways have been shown to be implicated in Eph signaling, including activation of the MAPK/ERK pathways by EphB2 receptor (Huusko, Ponciano-Jackson et al. 2004), inhibition of the Ras/ERK1/2 signaling cascade by EphA2, and phosphorylation of Src kinases and Akt by EphA2 and EphA4 (Guo, Miao et al. 2006).

Various human tissues differentially express the Eph/ephrin family (Hafner, Schmitz et al. 2004; Fox, Tabone et al. 2006; Hafner, Becker et al. 2006). Eph involvement in developmental processes, especially in embryonic development, vasculature and nervous system formation has been reported (Kullander and Klein 2002; Himanen and Nikolov 2003).

A growing body of evidence currently indicates that *Eph* gene family also plays an important role in carcinogenesis and tumor progression in many types of cancer (Andres, Reid et al. 1994; Walker-Daniels, Coffman et al. 1999; Liu, Ahmad et al. 2002; Kinch, Moore et al. 2003; Fang, Brantley-Sieders et al. 2005; Noren, Foos et al. 2006; Merlos-Suarez and Batlle 2008). Eph receptors were reported to have both tumor promoting activity, by being highly expressed in different human cancers, (i.e. breast, colon, melanomas, lung and prostate cancer) (Zelinski, Zantek et al. 2001; Surawska, Ma et al. 2004; Nakada, Drake et al. 2006; Foubert, Silvestre et al. 2007), and suppressing activity by acting as tumor suppressors (Batlle, Bacani et al. 2005; Noren, Foos et al. 2006). Some tumors are characterized by loss of expression of Eph receptors (EphB2 and B4 receptors in colorectal cancer and EphB6 receptor in breast cancer) (Alazzouzi, Davalos et al. 2005; Davalos, Dopeso et al. 2006; Fox and Kandpal 2006). Gene promoter methylation of *epha2*, *epha3*, *epha7*, *ephb2*, *ephb4* and *ephb6* was found in several human solid tumors, including breast, colorectal and prostate cancer, suggesting that an aberrant CpG island methylation, located in the promoter region of tumor related genes, may determine inactivation (Dottori, Down et al. 1999; Alazzouzi, Davalos et al. 2005; Wang, Kataoka et al. 2005; Davalos, Dopeso et al. 2006; Fox and Kandpal 2006; Nosh, Yamamoto et al. 2007). In addition, epigenetic silencing by hypermethylation

of *EPH/EPRIN* genes seems to contribute to the pathogenesis of acute lymphoblastic leukemia, where *ephb4* acts as a tumor suppressor (Kuang, Bai et al. 2010).

In breast cancer, *EphA2* appears to be important for the progression of the tumor from noninvasive to an invasive phenotype (Fox and Kandpal 2004), potentially via ErbB2 signaling (Brantley-Sieders, Zhuang et al. 2008). Fu et al. demonstrated that *EphA5* is frequently down regulated in breast cancer cell lines and tumor tissues *via* aberrant methylation of its promoter, a finding significantly associated with the clinico-pathologic tumor grade and metastasis to the lymph nodes (Fu, Wang et al. 2010). The authors also reported the presence of a dense GpG island with transcriptional and translational start sites at the 5' end of the *EphA5* gene. Such molecular architecture is entirely consistent with that described for other genes, known as targets for epigenetic silencing during tumorigenesis (Feltus, Lee et al. 2003), an early and frequent event in the development of breast cancer (Lehmann, Langer et al. 2002).

Altogether, these reports suggest that methylation of Eph genes might be used as a potential marker for cancer diagnosis and prognosis and underscore the need for further studies that better define their translational potential.

7. AXL receptor family

The Axl receptors belong to the TAM (Tyro-Axl-Mer) receptor tyrosine kinases family (Lai and Lemke 1991; O'Bryan, Frye et al. 1991). Axl is a 140-kDa protein, with an extracellular region comprised of two immunoglobulin-like domains and fibronectin type III repeats, a transmembrane region and a cytoplasmic domain with kinase activity (O'Bryan, Frye et al. 1991). There are two ligands for Axl: protein S and growth-arrest specific 6 (Gas6), the latter binding with higher affinity to Axl (Stitt, Conn et al. 1995; Varnum, Young et al. 1995). The main downstream Axl signaling is triggered through the phosphatidylinositol 3 kinase (PI3K) pathway (Collett, Sage et al. 2007), although in some circumstances the Janus kinase-signal transducers and activator of transcription (STAT) (Rothlin, Ghosh et al. 2007) or the p38 mitogen-activated protein kinase pathway (Allen, Linseman et al. 2002) may also be induced. The Axl receptor also cooperates with the cytokine receptor signaling network in order to regulate many biologic functions (Budagian, Bulanov et al. 2005; Gallicchio, Mitola et al. 2005) like cell survival, proliferation, adhesion and migration (Hafizi and Dahlback 2006). It contributes to vascular smooth muscle homeostasis and regulates endothelial cell migration and vascular network formation (Korshunov, Mohan et al. 2006; Collett, Sage et al. 2007).

Axl is overexpressed in several human cancers (Craven, Xu et al. 1995; Ito, Ito et al. 1999; Berclaz, Altermatt et al. 2001; Sun, Fujimoto et al. 2004) where it plays important roles in tumor angiogenesis (Holland, Powell et al. 2005; Li, Ye et al. 2009) and metastasis as demonstrated by studies in lung (Shieh, Lai et al. 2005), prostate (Sainaghi, Castello et al. 2005), breast (Meric, Lee et al. 2002), gastric (Wu, Li et al. 2002) and adrenal cell carcinomas (Chung, Malkowicz et al. 2003), as well as glioblastomas (Hutterer, Knyazev et al. 2008). Interestingly, Axl overexpression was reported to induce Imatinib resistance in gastrointestinal stromal tumors (Mahadevan, Cooke et al. 2007), Herceptin resistance in breast cancer (Liu, Greger et al. 2009) or chemotherapy resistance in acute myeloid leukemia (Hong, Lay et al. 2008), supporting its applicability as a potential therapeutic biomarker.

The invasive capacity of cancer cells was shown to be reduced by downregulation of Axl expression, as evidenced in breast and lung cancer (Holland, Powell et al. 2005; Shieh, Lai et al. 2005). Liu R et al. recently showed that Axl is induced in Kaposi sarcoma and Kaposi sarcoma herpesvirus (KSHV) transformed endothelial cells, but also in Kaposi sarcoma cell lines lacking KSHV (Liu, Gong et al. 2010).

Axl is transcriptionally regulated by the Sp1/Sp3 transcription factors and further controlled by CpG island methylation (Mudduluru and Allgayer 2008). Mudduluru et al. showed that transactivation of Axl gene is controlled by MZF1, which induces migration, invasion and in vivo metastasis formation (Mudduluru, Vajkoczy et al. 2010). In a subsequent study, focused on the epigenetic regulation of Axl at post-transcriptional level by micro-RNAs, the same authors showed that three micro-RNAs, miR-34a, miR-199a and miR-199b were frequently methylated and that their expression levels inversely correlates with Axl expression in three types of cancer: non-small cell lung cancer, colorectal cancer and breast cancer (Mudduluru, Ceppi et al. 2011).

TAM receptor inhibitions in animal xenograft tumor models of glioblastoma and breast cancer have provided preliminary validation of this receptor family as a cancer therapy target and further translational studies are needed in order to fully establish its potential.

8. ROR receptor family

Two human Ror RTK-encoding genes, *hRor1* and *hRor2*, the first Ror family members, and two rat partial complementary DNAs (cDNAs), *rRor1* and *rRor2* were identified nearly 10 years ago (Masiakowski and Carroll 1992). Ror family members have a conserved domain structure; the extracellular regions contain immunoglobulin (Ig), cysteine-rich (CRD) and kringle domains, all of which are thought to mediate protein-protein interactions. The intracellular domain of Rors contains a tyrosine kinase domain, two regions rich in serine and threonine separated by a region rich in prolines. There is a high similarity between the two Ror proteins Ror1 and Ror2 from each species. For instance, hRor1 and hRor2 have a 58% overall amino acid identity and 68% amino acid identity within the kinase domains (Forrester 2002).

ROR1 belongs to the RTK family of orphan receptors connected with muscle specific kinase and neurotrophin receptors (Masiakowski and Carroll 1992; Valenzuela, Stitt et al. 1995; Glass, Bowen et al. 1996). ROR has a predicted 937 amino acids sequence including an Ig-like domain, cysteine-rich domain, kringle domain, tyrosine kinase domain and proline-rich domain (Yoda, Oishi et al. 2003).

An alternative spliced form of *hRor1*, called truncated *hRor1* (Reddy, Phatak et al. 1996) has been reported in fetal and adult central nervous system, and was identified in human leukemia and lymphoma cell lines, as well as in a variety of neuroectodermal cancers. However, the function and significance of truncated hRor1 remain unclear (Reddy, Phatak et al. 1996; Forrester 2002).

Although ROR1 is situated on chromosomal region 1p31.3, which is a region where chromosomal aberrations are not frequently found in hematological malignancies, results from gene expression profiling studies show a 43.8-fold increase of ROR1 in chronic lymphocytic leukemia (CLL) cells (Klein, Tu et al. 2001). Furthermore, activation of NF- κ B in

CLL cells give rise to a functionally active ROR1 protein via Wnt5a and nonclassical Wnt-signaling pathway, supporting a role for ROR1 in the pathogenesis of CLL (Fukuda, Lu et al. 2004). Due to the high level of Ror1 surface expression in leukemic cells and its absence in normal (nonactivated) blood leukocytes (Daneshmanesh, Mikaelsson et al. 2008), ROR1 is now considered a candidate structure for targeted therapy, including monoclonal antibody-based therapies (Reddy, Phatak et al. 1996; Daneshmanesh, Mikaelsson et al. 2008). However, the exact mechanisms behind ROR1 gene regulation are yet to be fully delineated. Despite the recent evidence from fluorescence *in situ* hybridization (FISH) studies (Daneshmanesh, Mikaelsson et al. 2008) showing that the expression of RORr1 may not be related to genomic aberrations but rather epigenetic regulation, further evidence is needed.

Receptor tyrosine kinase-like orphan receptor 2 (ROR2), a transmembrane protein, is a member of a conserved family of tyrosine kinase receptors implicated in many developmental processes, including chondrogenesis (Sammar, Stricker et al. 2004), osteoblastogenesis (Liu, Bhat et al. 2007) and neural differentiation (Matsuda, Nomi et al. 2001). ROR2 mutations in humans result in dominant brachydactyly type B and Robinow syndrome (Ali, Jeffery et al. 2007; Lara, Calvanese et al. 2010).

ROR2 exerts its role in cell differentiation primarily via the Wnt signalling pathway (Angers and Moon 2009), employed by several extracellular effectors, membrane proteins, intracellular signal transducers and nuclear gene regulators that transmit extracellular signals to the nucleus as precise instructions for regulating specific genes (Aguilera, Munoz et al. 2007). Canonical Wnt is the signalling pathway involving β -catenin. Beta catenin-independent signals can also be induced by Wnt effectors via the non-canonical Wnt signalling pathway. The manner in which ROR2 realises its primary role, to mediate WNT5A signals within the Wnt signalling pathway, is still unclear (Lara, Calvanese et al. 2010). It was initially demonstrated in 293 cells that ROR2 mediates WNT5A-dependent inhibition of canonical Wnt signalling downstream of β -catenin, at the level of TCF-mediated transcription (Mikels and Nusse 2006). Subsequently it was shown that ROR2 mediates WNT5A dependent JNK (c-JUN NH₂-terminal protein kinase) activation and regulates convergent extension movements in *Xenopus* gastrulation (Schambony and Wedlich 2007) while in osteoblastic cells it enhances WNT1 and antagonise WNT3 activities (Billiard, Way et al. 2005). The inhibition of the β -catenin-dependent Wnt signalling pathway is mediated by ROR2 (Billiard, Way et al. 2005; Mikels and Nusse 2006; MacLeod, Hayes et al. 2007). Furthermore, by promoting constitutive Wnt signaling, the aberrant epigenetic repression of other Wnt inhibitors such as WIF-1, DKK1, SFRP1 and SFRP2 directly promotes tumourigenesis in colon cancer cells (Suzuki, Gabrielson et al. 2002; Mazieres, He et al. 2004; Aguilera, Fraga et al. 2006; Aguilera, Munoz et al. 2007). Inhibitor of the canonical Wnt signalling pathway in certain molecular contexts (Mikels and Nusse 2006), the ROR2 extracellular ligand WNT5A, is also aberrantly repressed by promoter hypermethylation in acute lymphoblastic leukaemia (Roman-Gomez, Jimenez-Velasco et al. 2007) and in colon cancer (Ying, Li et al. 2008), and its absence is tumourigenic in these tumour types.

ROR2 positively modulates Wnt3a-activated canonical signaling in the H441 lung carcinoma cell line (Li 2008). The Wnt signalling pathway is essential to cell differentiation and cancer. A primary mechanism of colon cancer development is the genetic and epigenetic changes of components of the canonical Wnt signalling pathway (Aguilera, Munoz et al. 2007). ROR2 is

overexpressed in oral (Kobayashi, Shibuya et al. 2009) and renal cancer (Wright, Brannon et al. 2009) and in osteosarcoma (Enomoto, Hayakawa et al. 2009). In osteosarcoma cells, the same suppressed expression of ROR2 (or its extracellular effector, WNT5A) diminishes invadopodium formation and inhibits cell invasiveness (Enomoto, Hayakawa et al. 2009). These studies underline the complex role of ROR2 in cancer and its function both as a promoter and suppressor of tumor formation, depending on the tumour type and molecular context.

Furthermore, ROR2 can mediate WNT5A-dependent activation of JNK, a member of the non-canonical Wnt pathway in mice (Oishi, Suzuki et al. 2003; Schambony and Wedlich 2007) and it can govern the WNT5A-dependent inhibition of canonical Wnt signalling downstream of β -catenin stabilization (Mikels and Nusse 2006). Considering the tumour type, ROR2 signals can therefore reveal a preference for β -catenin/TCF-dependent genes or for non-canonical Wnt pathways. There are two possibilities which support the evidences: firstly, in renal cancer and osteosarcoma activation of the non-canonical Wnt signalling kinase JNK mediates the pro-tumourigenic role of ROR2 (Enomoto, Hayakawa et al. 2009; Wright, Brannon et al. 2009) and secondly, restoration of ROR2 activity increased the inhibition of β -catenin reporter genes in colon cancer cells with constitutive Wnt signalling activity (MacLeod, Hayes et al. 2007; Lara, Calvanese et al. 2010).

As suggested for ROR2, WNT5A might have both a tumour-promoting and suppressing role. A tumour-suppressing effect has been reported in many studies, and it is downregulated in a number of cancers such as colorectal and ductal breast cancer, neuroblastoma and leukaemia (McDonald and Silver 2009). It was shown that WNT5A repression in colon and haematopoietic tumours is intervened by aberrant promoter hypermethylation (Roman-Gomez, Jimenez-Velasco et al. 2007; Ying, Li et al. 2008). WNT5A also reveals a tumour-promoting role in diseases such as non-small-cell lung cancer, melanoma, breast, gastric, pancreatic and prostate cancers (McDonald and Silver 2009). ROR2 knockout mice phenocopy most of the alterations seen in the WNT5A knockout mice, further supporting the intimate association between ROR2 and WNT5A and their complementary roles in cancer. While ROR2/WNT5A epigenetic downregulation would benefit tumours, such as colon and haematopoietic cancers typically driven by canonical Wnt signaling, their upregulation could be advantageous to cancers which are driven by non-canonical Wnt signaling (Lara, Calvanese et al. 2010).

Ror2 repression by aberrant promoter hypermethylation was reported in human colon cancer where epigenetic-dependent loss of ROR2 can promote tumour growth in colon cancer cells. Furthermore, ROR2 was reported to be overexpressed and have oncogenic properties in other tumour types such as oral cancer (Kobayashi, Shibuya et al. 2009), renal cancer (Wright, Brannon et al. 2009) and osteosarcoma (Enomoto, Hayakawa et al. 2009).

9. RET receptor family

Ret gene encodes for a receptor tyrosine kinase shared by four ligands, all members of the glial cell-derived neurotrophic factor (GDNF) family with important roles in neuronal survival: GDNF, artemin (ARTN), neurturin (NRTN) and persephin (PSPN) (Sariola and Saarma 2003). Signaling through RET is required for the development of the enteric nervous system, metanephric kidney and for the process of spermatogenesis (Schuchardt, D'Agati et

al. 1994; Meng, Lindahl et al. 2000). The human *RET* gene is localized on chromosome 10 (10q11.2) and contains 21 exons (Ceccherini, Bocciardi et al. 1993). Nine hundred base pairs within the promoter region of the *Ret* protooncogene contains 95 5'-CG-3' dinucleotide pairs, suggesting that *Ret* transcriptional activity might be regulated by DNA methylation (Munnes, Patrone et al. 1998).

Mutations of *Ret* are frequently reported in thyroid carcinoma and in Hirschsprung's (HSCR) disease (Pelet, Attie et al. 1994). Germline mutations in *Ret* induce constitutive RET protein activation and are the cause of multiple endocrine neoplasia type 2 (MEN 2). Also, *Ret* rearrangements were identified as a frequent pathogenic event that occurs in papillary thyroid carcinoma (PTC) (Jhiang, Sagartz et al. 1996).

The DNA methylation profile of *Ret* gene promoter was for the first time addressed in human HSCR disease (Munnes, Patrone et al. 1998). By bisulfite sequencing, Munnes, M., et al., reported that the *Ret* promoter is completely lacking 5-mC in both, malignant and normal samples (Munnes, Patrone et al. 1998). However, the methylation profile of the genomic region upstream of the *Ret* promoter indicated methylation in some CG sequences, with consequences on promoter activity (Munnes, Patrone et al. 1998). Recent investigations on the DNA methylation profiles at the *Ret* locus in medullary thyroid carcinoma identified a significantly lower degree of methylation in tumor cells compared with normal thyroid tissues (Angrisano, Sacchetti et al. 2011), providing supporting evidence for *Ret* epigenetics in thyroid cancer.

Furthermore, both histone and DNA methylation modifications seem to contribute to retinoic acid (RA)-mediated RET activation in neuroblastoma cells (Angrisano, Sacchetti et al. 2011) where several changes in methylation and acetylation profile of the core histones H3K4, H3K9, H3K9 and H3K27 within the *Ret* promoter region trigger modifications of *Ret* transcription. In addition, *in vitro* treatment of neuroblastoma cells (SK-N-BE) with a demethylating agent, seems to trigger an increase of RET expression even in the absence of RA (Angrisano, Sacchetti et al. 2011), indicating the possibility of using the methylation state of *Ret* proto-oncogene as a tumoral prognostic marker in cancer and raising the prospect of employing demethylating drugs in cancer therapy.

10. VEGF receptor family

Vascular endothelial growth factor receptors - 1 (VEGFR-1) and - 2 (VEGFR-2) play a critical role in physiologic and pathologic angiogenesis, including that associated with cancer (Carmeliet and Jain 2000; Ferrara 2005). Moreover, the expression of VEGF, VEGFR-1 and VEGFR-2 was reported in many solid tumors (Smith, Baker et al. 2010) of the colon (Kobayashi, Sugihara et al. 2008), ovary (Boocock, Charnock-Jones et al. 1995), breast (de Jong, van Diest et al. 2001), lung (Takahama, Tsutsumi et al. 1999) and prostate (Ferrer, Miller et al. 1999).

The 5'-flanking region of the VEGFR-1 gene contains a CpG island, where several putative transcription factor binding sites such as a TATA box and a binding protein/activating transcription factor (CREB/ATF) element have been described (Morishita, Johnson et al. 1995). In 2003, Yamada et al. reported an aberrant promoter methylation of the 5' region of the VEGFR1 detected by cDNA microarray screening analysis in prostate cancer cell lines compared to primary prostate samples (Yamada, Watanabe et al. 2003). The VEGFR-1

promoter was methylated in 38.1% of primary prostate cancer samples, in contrast to results from benign prostate samples where no promoter methylation could be detected. These results demonstrate that promoter methylation of VEGFR-1 plays a key role in silencing of this gene in prostate cancer cells (Yamada, Watanabe et al. 2003). However, the VEGFR-1 promoter methylation status was not elucidated in other cancer types. In 2009, Kim et al. evaluated the VEGF and VEGFR genes promoter methylation in different cancer cell lines and primary solid cancers (Kim, Hwang et al. 2009). While the VEGF promoter was not methylated in any of the cancer cell lines tested (colon, stomach, lung, melanoma, breast or thyroid cancers) VEGFR-1 as well as VEGFR-2 showed variable hypermethylation in the tested cancer cell lines, with an increased frequency in the stomach and colon cancer (Kim, Hwang et al. 2009). The study also showed a negative correlation between promoter methylation and expression of VEGFR genes, providing new insight on the epigenetic mechanisms underlying VEGFR expression in tumor tissues.

11. PDGFR family

Platelet-derived growth factor family includes four ligands: PDGFA, PDGFB PDGFC and PDGFD that regulate cell proliferation, cellular differentiation, cell growth, development and many diseases including cancer. The PDGFs bind to the protein tyrosine kinase receptors PDGF receptor- α and - β . These two receptor isoforms dimerize upon binding the ligand dimer, leading to three possible receptor combinations: - $\alpha\alpha$, - $\beta\beta$ and - $\alpha\beta$.

PDGFR α plays an important role in the normal development of neurons as well as in the pathogenesis of different disorders of the central nervous system. PDGFs and PDGFR α are coexpressed in human gliomas and glioma cell lines (Hermanson, Funa et al. 1992; Lokker, Sullivan et al. 2002). On the other hand, PDGFR α was reported to be overexpressed in malignant gliomas (Fleming, Saxena et al. 1992; Joensuu, Puputti et al. 2005; Carapancea, Cosaceanu et al. 2007) which leads to cell proliferation, invasion, and resistance to apoptosis. PDGFR activation initiates the signaling cascades that comprise the Ras/Raf/MAPK and PI3K/AKT pathways (Dong, Jia et al. 2011).

Several other neoplasia have also been associated with dysregulated expression of PDGFR α , in particular ovarian cancer (Lassus, Sihto et al. 2004), osteosarcoma (Sulzbacher, Birner et al. 2003), breast cancer (Carvalho, Milanezi et al. 2005) and laryngeal small cell carcinoma (Kanazawa, Nokubi et al. 2011). Following their initial studies showing that the human PDGFR α contains 10 polymorphic sites that give rise to 5 haplotypes, H1, H2a, H2b, H2g and H2d (Joosten, Toepoel et al. 2001), Joosten and colleagues later reported data on PDGFR α haplotype promoter's modifications and its consequences for the genetic predisposition of individuals to develop glioblastoma multiforme (Toepoel, Joosten et al. 2008). These studies show that H1 allele has a low activity in glioblastoma cell lines and is associated with allele-specific DNA methylation and histone deacetylation. Furthermore, epigenetic repression causes a low-activity of PDGFR α H1 allele during glial development and provides a reduced risk of glioblastoma development (Toepoel, Joosten et al. 2008).

12. Conclusion

Tumors are characterized by acquired somatic mutations and epigenetic alterations in genes that are crucial for differentiation, proliferation and survival pathways. Receptor tyrosine

kinases and their downstream signalling pathways play key roles in cancer development and are widely studied for their potential as therapeutic targets. It is increasingly evident that deciphering the mechanisms of RTK gene regulation in cancer is essential for the future development of new and improved therapies. Cancer epigenetics is one of the most rapidly expanding fields and current comprehensive epigenomic approaches will likely lead to a better understanding of the epigenetic regulations of RTK genes and their roles in proliferation, differentiation and cell growth and will open the door for the development of new treatment strategies based on these mechanisms. The advent of sensitive technology and the increasing evidence from recent studies provide a solid rationale for future exploration of epigenetics in mainstream oncology. Furthermore, the integration of epigenetics with data from genomics and transcriptomics will dramatically increase our understanding of tumorigenesis and will potentially yield better biomarkers for early detection, prognosis and therapy responses. The heterogeneity and genetic complexity of tumors is daunting, but the improvement in our knowledge of the pathogenetic mechanisms underlying RTK-induced transformation, coupled with the increasing availability of agents that target these pathways, offer unique opportunities for cancer research.

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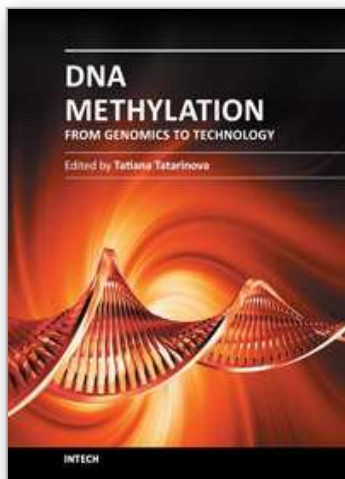
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Epigenetics is one of the most exciting and rapidly developing areas of modern genetics with applications in many disciplines from medicine to agriculture. The most common form of epigenetic modification is DNA methylation, which plays a key role in fundamental developmental processes such as embryogenesis and also in the response of organisms to a wide range of environmental stimuli. Indeed, epigenetics is increasingly regarded as one of the major mechanisms used by animals and plants to modulate their genome and its expression to adapt to a wide range of environmental factors. This book brings together a group of experts at the cutting edge of research into DNA methylation and highlights recent advances in methodology and knowledge of underlying mechanisms of this most important of genetic processes. The reader will gain an understanding of the impact, significance and recent advances within the field of epigenetics with a focus on DNA methylation.

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