

Chapter 4

Pathology and Molecular Biology



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Molecular Biology of Gastric Cancer

The Cancer Genome Atlas (TCGA) (2014), based on dysregulated pathways and candidate driver genes, has divided gastric cancer (GC) cases into four subtypes: Epstein-Barr virus (*EBV*)-infected tumors, microsatellite instability (*MSI*) tumors, genomically stable (*GS*) tumors, and chromosomal instability (*CIN*) tumors. The main characteristics of this classification are described in what follows.

According to the TCGA, around 50% of GC cases may be classified as chromosomally unstable, featuring marked aneuploidy, high somatic copy number alterations (SCNA), including focal amplification of receptor tyrosine kinases, such as *ERBB2*, *EGFR*, *ERBB3*, *FGFR2*, *MET*, *KRAS*, and *VEGFA*, as well as cell cycle mediators, such as *CCNE1*, *CCND1*, and *CDK6*, most of them amenable to targeted therapies. In addition, DNA hypomethylation and a high frequency of TP53 mutation may also be detected [1]. Another 9% of cases may be classified as positive for EBV, an alteration that may be accompanied by phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) mutations, DNA hypermethylation, including cyclin-dependent kinase inhibitor, also known as p16^{INK4A} (*CDKN2A*) silencing. Other alterations are amplifications of Janus kinase 2 (*JAK2*), *CD274* (also known as PD-L1), and programmed cell death 1 ligand 2, also known as PD-L2 (*PDCD1LG2*), that may be accompanied by mRNA increased expression, indicating implication of immune signaling. Another 20% of GC cases may be classified as microsatellite unstable tumors, showing elevated mutation rates, including mutations of genes encoding targetable oncogenic signaling proteins. In most of these cases, the mismatch repair defect is more likely due to an epigenetic hypermethylation in the *MLH1* promoter region. In addition, microsatellite unstable GCs are generally intestinal-type tumors, according to Lauren's classification. Finally,

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20% of cases may be classified as GS tumors, which are enriched for the diffuse histological variant and for mutations in *CDH1* (E-cadherin) or ras homolog family, member A (*RHOA*), or fusions involving Rho-family GTPase-activating proteins [1–3].

Another extensive molecular analysis was performed by the Asian Cancer Research Group (ACRG). Based on gene expression signatures, this group also identified four GC subtypes; however, they do not totally correspond to TCGA classification. In this analysis, GC samples were separated in microsatellite unstable (*MSI*) or microsatellite stable (*MSS*) tumors, which were further divided into tumors with epithelial-to-mesenchymal transition (*MSS-EMT*) signature, tumors with functional loss of TP53 (*MSS-TP53⁻*), and tumors with intact TP53 (*MSS-TP53⁺*). In this analysis, TP53 activation status was evaluated using a two-gene (*CDKN1A*, also known as p21, and *MDM2*) signature, in which a high score defines tumors with intact TP53 activity and vice versa [1, 2].

Following the ACRG expression signatures, the *MSI* subtype accounts for 22.7% of tumors and is enriched in intestinal tumors. This subtype is associated with a loss of expression of *MLH1*, elevated DNA methylation signature, and hypermutation, with alterations in genes such as *KRAS*, the PI3K-PTEN-mTOR pathway, *ALK*, *ARID1A*, and *PIK3CA*. Around 15% of the samples may be classified as being of the *MSS-EMT* subtype, which is enriched in diffuse Lauren histology. In *MSS-EMT*, loss of *CDH1* expression, as well as a lower number of somatic mutations and copy number variations, is more frequently found than in the other subtypes. Another 35.7% of tumors are *MSS-TP53⁻* and present low TP53 activity (low *CDKN1A* and *MDM2* scores) and a high TP53 mutation rate. The remaining 26.3% of cases are classified as *MSS-TP53⁺*, which present a lower TP53 mutation rate but a relatively higher prevalence of mutations in *APC*, *ARID1A*, *KRAS*, *PIK3CA*, and *SMAD4* than *MSS-TP53⁻*. In addition, in *MSS-TP53⁺*, EBV infection is more frequently detected [2].

Although a comparison between the TCGA and ACRG classifications shows similarities, it also reveals important differences. Among the similarities is the fact that there is an association between GC samples classified as *MSI* subtype, using both data sets. In addition, TCGA subtypes GS, EBV⁺, and CIN are enriched in samples classified as *MSS/EMT*, *MSS/TP53⁺*, and *MSS/TP53⁻*, respectively, according to ACRG. However, some differences may be detected when analyzing tumors with Lauren's diffuse histology. In addition, tumors classified as CIN, according to TCGA, may be classified in all four ACRG subtypes. Furthermore, even though EBV⁺ cases were more frequently detected in the *MSS/TP53⁺* subtype, they represented only a small proportion of samples (around 15%) from this subtype, suggesting that these correlations were also weak [1, 2].

Although GC studies have been increasing lately, few works have been dedicated solely to diffuse gastric cancer (DGC). According to the TCGA classification, the GS subtype is enriched in tumors with diffuse histology; these represent 73% of samples. However, among all tumor samples analyzed by this group, only 19% were characterized as GS. Considering all DGC included in the TCGA study, around 60% of DGCs were characterized as GS and the remaining 40% were classified in

each of the other subgroups, mainly CIN (28%), followed by MSI and EBV (6% each). In the ACRG analysis, the MSS-EMT subtype was enriched in samples with diffuse histology, which represented around 85% of MSS-EMT tumors. However, as reported earlier, only 15% of all the samples analyzed by the ACRG were classified as MSS-EMT. Hence, considering all DGC cases included in the analysis, 27% were classified as MSS-EMT, and the remaining diffuse tumors were characterized as MSS-TP53⁻ (31%), MSS-TP53⁺ (27%), and MSI (15%).

Considering both studies, a higher percentage of tumors with Lauren's diffuse histology was analyzed in the ACRG (45%) than in the TCGA (24%), and in the latter, diffuse tumors seemed less heterogeneous. In addition, *CDHI* and *RHOA* alterations, which correlate to the diffuse tumors and GS subtype (TCGA), were not frequent in the MSS-EMT subtype (ACRG). These differences suggest that the TCGA GS subtype is not equivalent to the ACRG MSS-EMT subtype [1, 2].

In the following paragraphs, we will describe the main characteristics of DGC, sometimes using data obtained for this specific Lauren's histological subtype and sometimes using data generated in GS tumors or MSS-EMT tumors, with the latter two subtypes mainly comprising DGC samples.

Genomic Alterations

Genes located at the same position (*locus*) in homologous chromosomes are known as alleles, and those alleles that are found more frequently in a population are known as wild-type alleles. When an alteration of the nucleotide sequence of the gene occurs, such as a substitution, an insertion, or a deletion, a new, mutant allele appears. These mutations may occur in germline cells, and therefore be hereditary, or they may occur sporadically in somatic cells, and in this case, they are not transmitted to the offspring. If the alteration is a small deletion or insertion, it may change the reading frame of the gene, and it is called a frame shift. This kind of mutation is likely pathogenic, leading to a dysfunctional protein and possibly to a disease. In addition, if a nucleotide substitution happens, it may create a stop codon (nonsense) or change a splicing sequence, which also gives rise to a dysfunctional protein. Otherwise, benign substitutions are silent and do not alter the protein function.

Copy number variations (CNVs) are alterations involving larger stretches of chromosomal DNA. When the alteration increases, decreases, or annihilates the number of copies of a gene (called amplification or deletion), it may lead to an overexpression, underexpression, or total absence of the protein, respectively. A mutation may also change the gene sequence in a chromosome, which is known as a translocation, usually engendering a chimeric protein whose function will be different from that of the original. Translocations may involve the breaking and rebinding of genes in the same chromosome or the exchange of DNA between different chromosomes. Moreover, many gene mutations are of unknown significance.

Let us first review somatic mutations associated with DGC and GS tumors. The genes most frequently mutated in DGC, according to COSMIC (Catalogue of

Table 4.1 Genes most frequently mutated in DGC

Gene	Name	Functional group	Role in carcinogenesis
<i>CDH1</i>	E-cadherin 1	Cell adhesion	Tumor suppressor
<i>RHOA</i>	Ras homolog family member A	Cytoskeleton and cell motility	Unknown (see section on <i>RHOA</i> pathway)
<i>TP53</i>	Tumor protein p53	Genome integrity	Tumor suppressor
<i>ARID1A</i>	AT-rich interaction domain 1A	Chromatin remodeling	Tumor suppressor
<i>PIK3CA</i>	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha	Receptor tyrosine kinase pathway	Oncogene
<i>KRAS</i>	Kirsten rat sarcoma viral oncogene homolog	Receptor tyrosine kinase/cell cycle	Oncogene
<i>CTNNB1</i>	Catenin beta 1	Cell adhesion/Wnt-signaling pathway	Oncogene

Somatic Mutation in Cancer), include *TP53* (39%), *CDH1* (23%), *ARID1A* (20%), *RHOA* (13%), *PIK3CA* (8%), and *SMAD4* (7,5%). Similarly, in GS tumors, somatic mutations are most frequently found in genes such as *CDH1* (cadherin 1), *RHOA*, *ARID1A*, *PIK3CA*, *TP53*, *KRAS*, and *CTNNB1*. Table 4.1 describes the main functions and roles of these genes in carcinogenesis [4]. Other whole-genome (exome) sequencing studies reported similar results [5, 6].

In GS tumors, *CDH1* somatic mutation is relatively frequent (37% of tumors, mainly missense), but generally it is not concomitant with *TP53* mutation [1]. In studies analyzing specifically DGC, the proportion of *CDH1* mutation varies between 23% and 33% and is significantly higher than in other histological and molecular subtypes. The most frequent type of mutation is missense, leading to a dysfunctional protein that impairs cell adhesion [1, 5, 6].

RHOA, involved in actin organization and cell migration, is another frequently mutated gene in GS tumors as well as in DGC. Notably, *RHOA* mutations are found only in DGC, in which its frequency varies between 10% and 25%. The most common alterations are missense, but whether those lead to a gain or loss of function remains unclear. Nevertheless, recent findings indicate that *RHOA* may be a driver mutation in the diffuse histological subtype [1, 5–7]. In addition, dysregulated RHO signaling may also be detected as interchromosomal translocations between Rho-family GTPase-activating proteins, such as *CLDN18* and *ARHGAP26* (GRAF) or *CLDN18-ARHGAP6*. Together, these mutually exclusive alterations may be found in 30% of GS tumors. Another chromosomal translocation described in DGC is *SLC34A2-ROSI*, which affects a gene that codes a receptor tyrosine kinase [1, 8].

Considering SCNA, the most frequently reported in GS tumors are focal amplifications targeting genes such as *VEGFA*, *GATA4*, *MYC*, *FGFR2*, *CD44*, 11q14.1, *KRAS*, 12q13.11, *MDM2*, 15q26.1, and Xq27.3. In addition, focal deletions targeting regions 2q32.1, 3p24.1, 4q25, *PTPRD*, *CDKN2A*, 18q23, Xq21.23, including genes localized at fragile sites such as *FAM190A*, *PDEA4D*, *IMMP2L*, *WWOX*, and *MACROD2*, were also described in GS tumors [1].

Epigenetics

The modification in the DNA sequence of oncogenes and tumor suppressor genes is well known and characterized in cancer. Additionally, chromatin structure and organization have a significant effect on gene expression. The study of heritable changes in gene expression that occur independently of changes in DNA sequence is called epigenetics. In the past decade, the role of epigenetic abnormalities in cancer pathogenesis has been extensively investigated.

The main epigenetic mechanisms include changes in DNA methylation profile, histone modifications, and abnormalities of microRNA expression or binding. In this section, the contribution of these alterations for DGC will be discussed.

DNA Methylation

The process of DNA methylation is the most studied epigenetic modification. It occurs in chromatin sequences rich in CpG dinucleotides, usually clustered in regions called CpG islands. The methylation status of a CpG island is associated with gene-expression variation. When a DNA region loses its methyl group or a methyl is added in a position usually unmethylated, this is called hypomethylation and hypermethylation, respectively. (Sharma et al. 2009). The enzymes responsible for the transfer of methyl groups to the DNA are called DNA methyltransferases (DNMT).

Cancer is known for presenting a conflicting epigenetic profile: global hypomethylation and gene-specific hypermethylation. Global hypomethylation is considered one of cancer's hallmarks and is believed to be associated with the disease by the mechanisms of chromosomal instability, reactivation of transposable elements, and loss of imprinting. Otherwise, hypermethylation of CpG islands in gene promoters is associated with gene silencing and loss of protein expression [9].

According to TGCA, GS and CIN tumors have similar hypermethylation profiles, which are less prominent than EBV and MSI subtypes. Nevertheless, when MSS non-EBV tumors are reclassified according to histological subtype, DGCs present higher rates of CpG island methylation, whereas intestinal tumors show a higher chromosomal instability index and more widespread demethylation of the genome [1, 5].

In DGC, the best characterized gene that undergoes promoter hypermethylation is *CDH1*, which codifies the cell-adhesion protein, epithelial cadherin (E-cadherin) 1. The methylation of *CDH1* promoter is largely found in gastric tumors and can lead to gene silencing and reduced protein expression. This is one of the possible mechanisms involved in the complete inactivation of the *CDH1* gene in hereditary DGC and sporadic DGC [10].

Considering DNA methyltransferases, some aberrant patterns have been described in gastric tumors. Overexpression of DNMT 1, 3A and 3B in stomach neoplastic tissue was reported in some studies, and seems to be associated with

clinicopathological features. DNMT3A levels were linked to tumor stage and lymph node metastasis, and higher levels of DNMT3B were related to lymph node metastasis. Although these findings represent an advance in epigenetic knowledge, the cause and consequences of this enzyme's expression is not fully understood. Therefore, the role of the DNMT family is likely extensive in gastric carcinogenesis, but the specific correlation with the diffuse subtype is yet to be investigated [11].

Histone Alterations

Histones are proteins that bind to DNA, providing stability to chromatin. The interaction between histones and DNA determines the accessibility of chromatin to the transcription apparatus. Generally, acetylated histones allow transcription factors to interact with chromatin, to induce DNA transcription, in contrast to methylated histones, which tend to decrease DNA transcription. In GC, methylation of histones, such as H3K27me3 and H3K9me3, are associated with poorer prognosis by inactivating tumor suppressor genes [11]. There is no described pattern of histone modification specific to DGC.

MicroRNA

MicroRNA (miRNA) constitutes another layer of gene-expression regulation. MiRNAs are small noncoding RNA sequences of approximately 22 nucleotides that may interact through base pairing with complementary sequences in the 3' untranslated region (3' UTR) of messenger RNAs (mRNAs) to target them for degradation and thereby prevent their translation. More than 1000 individual miRNAs have been identified, and each one can target a great number of different mRNAs. miRNAs can control cell proliferation, differentiation, and survival, among other processes, so changes in miRNA expression patterns may be involved in tumor development [12].

According to the TCGA, some miRNA (miR) such as miR-1, miR-133a-3p, miR-133b, miR-145-3p, miR-143-3p, miR-490-3p, let-7c-5p, miR-125b-2-3p, and miR-99a-5p are relatively more expressed in GS tumors, compared to the other subtypes. However, these same miRNAs are similarly expressed between GS tumors and gastric normal tissue [1].

In another study, the TCGA database was reevaluated to characterize miRNAs expressed in diffuse and intestinal histological subtypes. The miRNAs 100-5p, 195, let-7c-5p, 140, 99a-5p, and 125-b2-3p were correlated with the diffuse subtype, while miRNAs 210, 592, 130b, and 455 were associated with the intestinal subtype [1, 13]. The miRNAs 100-5p, 99a-5p, and 125-b2-3p may be involved in the regulation of hematopoietic stem cells by TGFbeta and Wnt signaling pathways. We have further explored this miRNA data to identify mRNA target candidates using miRWalk 2.0: a comprehensive atlas of predicted and validated miRNA-target interactions [14], followed by Toppgene suite, to perform gene list enrichment analysis and candidate gene prioritization [15]. Potential targets for miR let-7c-5p are genes such as

MAP3K1, *RANBP2*, *EIF2S2*, *CTPS2*, *ZNF341*, and *FNIP1*, and biological processes enriched in these genes are “de novo” CTP biosynthetic process, positive regulation of B-cell apoptotic process, positive regulation of protein complex assembly (*MAP3K1* and *FNIP1*), and regulation of pro-B cell differentiation; the targets for miR 99a-5p/100-5p are mainly *EMR2*, *USP12*, *HSD3B7*, *IMPDH1*, *TNFAIP8L1*, *C20orf194*, *CAND2*, *MYCBP*, *TRIB2*, *FOXJ3*, *RRN3*, *ICMT*, *ZZEF*, *SETD1B*, *KDM6B*, and *ALG13*, and enriched biological processes are the negative regulation of interleukin-T10 biosynthetic process (*TRIB2*), SCF complex assembly (*CAND2*), regulation of interleukin-10 biosynthetic process (*TRIB2*), and C-terminal protein methylation (*ICMT*); the targets for miR 125-b2-3p are *KCNT1*, *KLC1*, *RPL28*, *CCPG1*, *SLC35D2*, *PCMTD2*, and *NSFL1C*, and the biological process enriched are the regulation of Rho guanyl-nucleotide exchange factor activity (*CCPG1*), stress granule disassembly (*KLC1*), regulation of guanyl-nucleotide exchange factor activity (*CCPG1*), pyrimidine nucleotide-sugar transmembrane transport (*SLC35D2*), and organelle disassembly (*KLC1*, *RPL28*).

Gene and Protein Expression

A way to further improve the characterization of GC subtypes has been through differential gene expression analysis, especially through cDNA microarray, where the information coded by all transcribed genes may be considered.

Analysis of the TCGA database, using RNA seq data sets, revealed 40 differentially expressed genes that might classify the groups MSI, CIN, EBV, and GS. Among these genes, 10 were more expressed in GS tumors in relation to CIN, MIS, and EBV: *FLNC*, *HSPB6*, *ACTG2*, *CNN1*, *DES*, *HSPB7*, *LYOD1*, *MYH11*, *SYNPO2*, and *SYNM*. Using ToppGene analysis, biological process enriched in these genes were muscle contraction (*HSPB6*, *ACTG2*, *CNN1*, *DES*, *MYH11*, *SYNM*); regulation of system process (*HSPB6*, *CNN1*, *DES*, *HSPB7*); actin filament-based process (*FLNC*, *CNN1*, *DES*, *MYH11*); and intermediate filament cytoskeleton organization (*SYN*, *DES*). However, the expression of these genes was similar in the comparison between normal tissue and GS tumors. In addition, some genes were differentially abundant only between GS group versus adjacent normal tissue, some more expressed (*SFRP4*, *CLDN3*, *THBS4*, *THBS2*, *CST1*, *BGN*, *FNDCl*, *COL8A1*, *ASPN*) and others less expressed (*GKN1*, *GKN2*, *LIPF*, *PGC*, *TFF2*, *GIF*, *REG3A*, *PGA3*, *PSCA*, *CXCL17*) in GS tumors [1].

In another attempt to better classify the histological subtypes, it was shown that genes overexpressed in diffuse tumors code for proteins involved in extracellular matrix processes. In this work, thrombospondin 4 (THBS4), an important adhesive glycoprotein, was more expressed in diffuse than intestinal subtypes. In addition, using immunohistochemistry, it was shown that THBS4 may be detected specifically in the stromal compartment of diffuse tumors [16].

Further studies used microdissected diffuse-type GC, as compared to their corresponding noncancerous mucosae, to reveal differentially expressed genes

that might be involved in carcinogenesis and tumor progression. Genes more expressed in tumor samples included *COL3A1*, *TGFB1*, *SPARC*, *MSLN*, *FLJ20736*, *GW112*, *EST (AA430699)*, and *EST (AA143060)*. In addition, comparison of the expression profiles of the diffuse type with those of the intestinal type demonstrated 46 differentially expressed genes. Fourteen genes were more expressed in the diffuse type, including those encoding chaperones (*CCT3* and *TOR1B*) or associated with cell motility and cytoskeleton (*CD81* and *TUBA3*) and glycosylation (*RPN2*, *MGAT1*, and *MPI*). Another 32 genes were more expressed in diffuse-type tumors, such as those involved in signal transduction and transcriptional regulation (*RHBDL*, *SFRS8*, *MLL5*, *LDB3*, and *GFRA2*), nuclear transportation (*KPNB2* and *NUPI33*) and cell adhesion (*PSK-1*, *ITGB5*, *SRPX* and *IBSP*). In conclusion, this study identified genes that could distinguish different mechanisms underlying gastric carcinogenesis [17].

Proteomic analysis of GS tumors may also add some clues to tumor behavior. Increased protein expression of *CAVI*, *MYH11*, and *RICTOR* and reduced expression of *CTNNB1* (Catenin Beta 1), *CDH1*, and *MTOR* in GS tumors as compared to other subtypes of GC was described. Other proteins that may be less expressed in GS tumors, as compared with MSI-H, EBV e CIN subtypes, were *KIT*, *HSP70*, *MYC*, *PRKCA*, *PRKCA pS657*, *CCND1* (Cyclin D1), *EIF4EBP1 pS65*, *ACVRL1*, *BCL2*, *TUBA acetyl Lys40*, *CoO16A1*, *PKC-pan pS660*, *PEA15*, and *AKT* [1].

Finally, expression of some genes, such as *HER2*, are particularly important due to their clinical relevance. For example, *HER2-neu* overexpression, used to indicate trastuzumab treatment, is detected in only a small percentage of diffuse histology cancers, around 6%, in contrast with 32% of the intestinal histology cases [18].

Pathways

Hierarchical clustering of samples and pathways revealed that the GS subtype exhibited elevated expression of cell-adhesion pathways, including the B1/B3 integrins, syndecan-1-mediated signaling, and angiogenesis-related pathways in contrast to other subtypes (CIN, EBV, MSI), which exhibited elevated expression of mitotic network components such as *AURKA/B* and *E2F*, targets of *MYC* activation, *FOXO1* and *PLK1* signaling, and DNA damage response pathways [1].

Specifically, one molecule involved in the cell-adhesion pathway deserves further attention regarding its mechanism of regulation: E-cadherin.

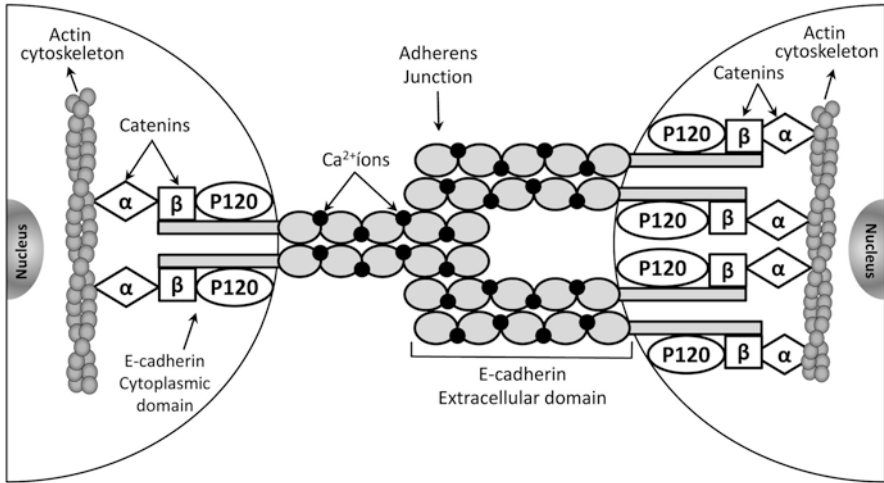


Fig. 4.1 Cell adhesion mediated by E-cadherin. E-cadherin homodimerizes in the presence of calcium ions and binds to other E-cadherin molecules from an adjacent cell through the extracellular domain; p120 catenin and β -catenin interact with the E-cadherin cytoplasmic domain. Subsequently, β -catenin interacts with α -catenin, which then anchors the structure to the actin cytoskeleton

E-Cadherin and Cell Adhesion

E-cadherin is encoded by the *CDH1* gene, which is located on chromosome 16q22.1 and is composed of 16 exons and 15 introns. E-cadherin belongs to the family of cell-adhesion molecules and plays a fundamental role in the maintenance of cell differentiation and the normal architecture of epithelial tissues [19].

E-cadherin is composed of three major structural domains: an extracellular domain, comprising five tandemly repeated domains, EC1–EC5; a single transmembrane domain; and a cytoplasmic domain. The extracellular domain is involved in cell adhesion, through homodimerization with cadherins from adjacent cells, in the presence of calcium ions. The cytoplasmic domain includes the juxtamembrane domain, which interacts with p120-catenin and the catenin-binding domain, which binds beta-catenin and gamma-catenin. In a second step, beta-catenin binds to alfa-catenin, which is anchored to the actin cytoskeleton, establishing the cadherin–catenin complex (Fig. 4.1). The stability of the cadherin–catenin complex and its linkage to actin filaments form the core of the adherens junction, which is vital in the inhibition of individual epithelial cell motility and in providing homeostatic tissue architecture [19].

E-Cadherin and the Wnt/Beta-Catenin Signaling Pathway

Wnt and cadherin pathways are important in the regulation of beta-catenin activity. Extracellular Wnt proteins bind to cell surface receptors of the Frizzled family, promoting beta-catenin translocation from the cytoplasm to the nucleus. In the nucleus, beta-catenin activates transcription factors, such as TCF and LEF, inducing the transcription of target genes involved in cell migration, cell proliferation, and apoptosis [20].

Beta-catenin can be found in the membrane, cytoplasm, or nucleus depending on the status of Wnt signals and the expression and distribution of E-cadherin. In most cases, overexpression of E-cadherin inhibits beta-catenin transcriptional activity. Contrariwise, when Wnt signaling is active or when E-cadherin is phosphorylated by a tyrosine kinase, it releases beta-catenin. The latter then accumulates in the cytoplasm and translocates to the nucleus, where it can then regulate the transcription [19, 21].

E-Cadherin and Epithelial-to-Mesenchymal Transition

There is accumulating evidence that epithelial–mesenchymal transition (EMT) is involved in GC aggressiveness. EMT is a biological process that allows a polarized epithelial cell (adherent cell) to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype, including enhanced migratory capacity, invasiveness, and elevated resistance to apoptosis [22]. When cells undergo EMT, they lose E-cadherin, dissolve cell adhesions, and are prone to invading adjacent tissues and metastasizing. Hence, maintaining adequate E-cadherin levels is an important mechanism to preserve tissue architecture and inhibit the invasion of adjacent tissues [23].

E-Cadherin Expression and Function

CDH1 may be considered a tumor suppressor gene, linked with human cancer susceptibility [24]. Consequently, abrogation of E-cadherin function, through genetic, epigenetic, or posttranslational mechanisms, may be a carcinogenic event. Besides gene mutations, other mechanisms that may be involved in *CDH1* downregulation include promoter methylation or upregulation of transcriptional repressors.

E-cadherin immunoreactivity is often reduced or lost in less differentiated and invasive diffuse carcinomas. E-cadherin aberrant immunoreactions have been observed significantly more frequently in the diffuse-type carcinomas (82.4%) in comparison to the intestinal-type carcinomas (31.6%), emphasizing the strong relation between Lauren's classification of gastric carcinomas and the immunohistochemical expression of the E-cadherin cellular adhesion molecule [25].

Germline and Somatic Mutations in *CDH1* Gene

Sporadic *CDH1* genetic and epigenetic alterations were described earlier in this chapter. In summary, 23–33% of DGCs present *CDH1* mutations, leading to defective cell-adhesion function, and epigenetic silencing of the gene promoter by methylation is very frequently associated with low E-cadherin expression [1, 5, 6, 10]. Hereditary genetic and epigenetic alterations in *CDH1* are discussed elsewhere (Chap. 5).

Transcriptional Regulation of E-Cadherin Expression

Transcriptional control is an essential regulatory mechanism of E-cadherin expression. The characterization of an E-cadherin promoter region revealed several potential proximal regulatory elements: a CCAAT box (–65), a GC-rich region (–30 to –58), and a palindromic element (–70 to –90), composed of adjacent E-boxes, flanked by four inverted nucleotides called Epal. The proximal CCAAT and GC-rich regions, which are recognized by constitutive AP2 and Sp1 transcriptional factors and CAAT-binding proteins, respectively, are required for basal E-cadherin expression [26].

A major breakthrough in understanding the regulation of E-cadherin transcription was the identification of several E-cadherin repressors. These transcriptional repressors, represented by the zinc finger factors Snail and Slug (another member of the Snail family), as well as by class I basic region helix–loop–helix (bHLH) factor E47, specifically bind to the E-boxes and overcome the positive effects of constitutive factors. In addition, another two factors of the zinc finger family, Zeb1 and Zeb2, have also been described as repressors of E-cadherin.

Functional characterization of Snail indicates that it does indeed act as a strong repressor of the E-cadherin promoter. Snail repressor activity apparently requires three E-boxes of the human promoter. Importantly, overexpression of Snail in epithelial cells produces a dramatic EMT and promotes the acquisition of migratory and in vitro invasive behavior [27]. E47 and Slug were also shown to behave as E-cadherin repressors and to induce EMT when overexpressed in epithelial cell lines [26].

Posttranslational Regulation of E-Cadherin Expression

E-cadherin cellular levels may also be regulated through posttranslational modifications, such as phosphorylation, glycosylation, and proteolytic processing. Some important players in this process are p120 catenin and Hakai. Binding of p120 catenin with the cadherin juxtamembrane domain stabilizes and suppresses cadherin endocytosis and promotes the formation of adherens junctions. Removal of p120 from the cadherin complex, via phosphorylation of p120, uncovers an adaptor

protein 2 (AP-2) binding motif, as well as a phosphorylation-dependent motif for the recruitment of the E3 ligase Hakai. AP-2 binding promotes clathrin-dependent endocytosis of E-cadherin, which can subsequently be recycled back to the membrane. Otherwise, the endocytosed E-cadherin may be a target of Hakai-induced ubiquitination followed by degradation in the proteasome [28].

RHOA Pathway

RHOA is a member of the RAS superfamily, which is known to be involved in cell proliferation. *RHOA* is a small GTPase, encoded by a gene in chromosome 3, and it is highly conserved in species over the course of evolution. It participates in numerous biological processes by functioning as a molecular switch in signal transduction cascades. Rho proteins promote actin polymerization and regulate cell shape, attachment, and motility. They are also involved with cell cycle progression.

Recently, *RHOA* mutations have been described by whole-genome sequencing in GC studies as being exclusive to DGC or GS and a possible new driver of this subtype of diseases. Most *RHOA* alterations occur in functional domains involved in GTP binding and interaction with effectors designed as hotspots. Whether these mutations promote gain or loss of *RHOA* function is not clear. The evidence of loss of heterozygosity and anoikis escape in cells with mutated *RHOA* indicates loss of function, while growth-promoting effects in cells and bioinformatic analysis showing activation of *RHOA* pathways with mutated gene variants suggests gain of function. These data may indicate that, even though mutant *RHOA* lose their GTP-binding capacity, they may acquire a new oncogenic activity, perhaps by an unidentified signaling pathway [7].

The *RHOA* signaling pathway may also be altered by a recently described chromosomal translocation. CLDN18-ARHGAP26 is a fusion protein recurrently screened in GC samples. ARHGAP26 negatively regulates *RHOA* activity via the GAP domain. Under the influence of CLDN18 promoter, ARHGAP26 inactivates *RHOA*, and as a result, the actin cytoskeleton and cell-to-cell adhesion are affected. Therefore, epithelial tissue is damaged and its cells may not be replaced, promoting gaps that enhance tissue injury and may eventually lead to GC [1].

The nature of interaction between *RHOA* and *CDH1* is of importance for understanding DGC molecular pathology. A missense mutation on the extracellular domain of E-cadherin is believed to be responsible for increased cell motility in a mechanism involving *RHOA* activation. E-cadherin mutants show reduced stability of E-cadherin/EGFR heterodimers. This results in their disruption and EGF activation of homodimers, which leads to *RHOA* activation and increased cell motility. These data give new insights into the understanding of mechanisms linking invasion and E-cadherin mutations in DGC [29].

These new findings place *RHOA* as an important candidate gene target for new therapies in DGC.

Survival According to Molecular Subtypes

The diffuse-type GC, according to Lauren classification and description, is associated with a poorer prognosis when compared to the intestinal subtype. This pattern has been confirmed as more specific markers of lower survival rates, such as the presence of signet-ring cells and poorly differentiated histology, were increasingly related to DGC.

More recently, molecular classification has added information regarding the prognostic value of DGC. In the TCGA study, the four molecular subtypes described, CIN, MSI, GS, and EBV, were not associated with significant differences in survival rates. However, ACRG data revealed that MSS-EMT patients (enriched in the DGC subtype) had the worst prognosis, after MSS-TP53⁻, MSS-TP53⁺, and MSI. However, in the ACRG classification, DGC was almost evenly distributed in all four subtypes. An evaluation of a larger number of DGCs might indicate differential prognosis associated with diverse mechanisms of carcinogenesis [1, 2].

In summary, important new data are beginning to unravel the carcinogenic process in DGC, further indicating that cell-adhesion and extracellular matrix processes may be disrupted in DGC. Although the incidence of the intestinal subtype has been decreasing over the years, the same is not observed for diffuse tumors. Hence, additional research is needed to unravel potential targets of therapy in DGC.

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