Molecular Pathology of Gastrointestinal Cancer

Guest Editors: Rhonda K. Yantiss, Alyssa M. Krasinskas, Wade Samowitz, and Brian Rubin
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Serrated Polyposis: An Enigmatic Model of Colorectal Cancer Predisposition

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Serrated polyposis has only recently been accepted as a condition which carries an increased personal and familial risk of colorectal cancer. Described over four decades ago, it remains one of the most underrecognized and poorly understood of all the intestinal polyposes. With a variety of phenotypic presentations, it is likely that serrated polyposis represents a group of diseases rather than a single entity. Further, neoplastic progression in serrated polyposis may be associated with premature aging in the normal mucosa, typified by widespread gene promoter hypermethylation. From this epigenetically altered field, arise diverse polyps and cancers which show a range of molecular features. Despite a high serrated polyp count, only one-third of colorectal cancers demonstrate a BRAF V600E mutation, the molecular hallmark of the canonical serrated pathway, suggesting that though multiple serrated polyps act as a marker of an abnormal mucosa, the majority of CRC in these patients arise within lesions other than BRAF-mutated serrated polyps.

1. Introduction

Serrated polyposis [1], a condition also known as hyperplastic polyposis, was described in the early seventies [2] and remains to the present day the most under-recognized and least understood of the colorectal polyposes. Its defining characteristic, specifically the presence of numerous colorectal epithelial polyps with serrated architecture (Figure 1, Text Box 1), placed it, until relatively recently, among conditions without significant clinical consequence, based upon the perception that all serrated polyps were innocuous. For decades, the malignant transformation of conventional adenomas was considered to be the single mechanism underlying the genesis of colorectal cancer (CRC) [3]. In the late nineties, a number of important observations set in motion a major paradigm shift in the way the initiation and progression of CRC were viewed. These observations carefully detailed at a molecular level that some serrated polyps may act as the precursor lesions in an alternative developmental pathway for CRC, existing alongside the traditional adenoma-carcinoma sequence [4]. As with the histological observations suggesting that a subset of serrated polyps may develop features associated with malignancy [5–7], clear molecular evidence for the malignant transformation of serrated polyps was also first observed in a patient with serrated polyposis [8], suggesting that this condition could serve as a model for the malignant conversion of serrated polyps, a mechanism which has become known as the serrated pathway. Today the clinical significance of serrated polyposis rests upon consistent observations in relatively large studies of an increased personal and familial risk of CRC [9–12]. To include serrated polyposis as a CRC predisposition is a concept whose time has come [13].

2. Definition and Features

Clinical criteria for the recognition of serrated polyposis were first established in 2000 [14] and were proposed for
When establishing the serrated polyp count for an individual, Higuchi and Jass have suggested that the polyp count be cumulative over time [18]. Serrated polyposis affects both sexes and is most likely to be identified in persons aged between 55 and 65 years. It may become apparent considerably earlier [7, 19–21], particularly if symptoms such as bleeding, bowel habit alterations, and abdominal pain result from the polyp burden or advanced neoplasia. The range of ages at presentation in the literature varies from 11 to 83 years [7, 19–21].

Estimates of the prevalence of serrated polyposis suggest that it is relatively rare. A large population-based screening trial of over 40,000 asymptomatic patients aged 55–64 years prospectively identified serrated polyposis at a rate of 1 in 3000, with 50% of these demonstrating at least one synchronous conventional adenoma [22]. Rubio et al. reported that only 10 cases were observed in a 1026-bed Scandinavian hospital over a 16-year period [23], whilst Leggett et al. identified 12 cases from a similar institution during a 5-year period [24]. A family history of CRC is a relatively frequent finding with figures of up to 59% reported [25–27], though other publications suggest that this is rare [28, 29]. A recent large multicentre study from The Netherlands has estimated the risk to first-degree relatives of CRC to be approximately fivefold that of the general population adjusted for demographic variables: RR 5.4 (95% CI 3.7 to 7.8) [10]. Studies of ethnicity suggest that, in contrast to conditions such as FAP and Lynch syndrome which can occur in many ethnic groups, serrated polyposis is a condition largely of north-western Europeans. Observations from a multiethnic New Zealand gastroenterology service demonstrated that, in a 24-case series of serrated polyposis patients, all cases were derived from the European component, despite only 46% of the attending patients having European ancestry [17]. Buchanan et al. reported a prevalence of 95% northern Europeans in a case series of 126 serrated polyposis patients [11]. Similarly, Kalady et al. reported that, in a large series of serrated polyposis patients (n = 115) collected in Ohio, 97% were white [27]. The fundamental defect in serrated polyposis has yet to be elucidated and may involve defects in inflammation and/or apoptosis. The involvement of widespread DNA methylation in the normal mucosa of patients with this condition [30] suggests deregulation of an epigenetic control mechanism, either directly or as a consequence of upstream genetic events.

3. History of Its Recognition as a Colorectal Cancer Predisposition

Described in some earlier reports primarily in order to distinguish it from FAP, serrated polyposis was originally considered to have no important clinical consequences [29]. However, today it is recognized as a condition with substantial risk of CRC. The early literature contains much to interest those who study serrated polyposis, and the serrated pathway to carcinoma in general. Serrated polyposis was described as early as 1970 by Goldman et al. [2]. In this case report,
Epithelial polyps with serrated architecture arising in the large intestine were until very recently collectively known as hyperplastic polyps. A modern classification has now been proposed which uses the descriptive umbrella term serrated polyps for all epithelial polyps with serrated architecture and the term hyperplastic polyps for the subset of small common lesions without evidence of abnormal proliferation. A detailed description of the WHO classification of serrated polyps is given in Section 3 of this paper.

Text Box 1: Serrated polyps or hyperplastic polyps? [1].

- (1) At least five serrated polyps proximal to the sigmoid colon with two or more of these being >10 mm.
- (2) Any number of serrated polyps proximal to the sigmoid colon in an individual who has a first-degree relative with serrated polyposis.
- (3) >20 serrated polyps of any size, but distributed throughout the colon.

Text Box 2: Clinical criteria for the identification of serrated polyposis [1].

a 42-year-old man presented with 30 serrated polyps ranging in size between 0.75 and 1.5 cm. Of particular interest was the observation of adenomatous transformation within the polyps, a concept now readily accepted, but somewhat controversial in those earlier times. Despite several reports relating serrated polyps to the development of adenomatous change, villous components, and even adenocarcinoma [31–33], the bulk of investigations published around this time returned a classification of nonneoplastic for serrated polyps [3, 34–36].

In 1978, Cooke described serrated polyposis as a variant of FAP [5]. A further report describing cancer and dysplasia in a background of serrated polyposis was presented in 1979 [37]. In this publication, the authors, whilst suggesting that hyperplastic polyps were “benign, nonneoplastic proliferations which unlike tubular and villous adenomas did not predispose the patient to colonic cancer,” went on to demonstrate a case of serrated polyposis where hyperplastic adenomatous transformation and cancer “had probably occurred,” and recognized that cancer could be associated with unusual cases of multiple hyperplastic polyps [6]. In 1980, seven cases of serrated polyposis were recorded from a London hospital [29], and this became an influential landmark paper in the confusion surrounding serrated polyposis. Six of the seven cases were male, and there was an average age at presentation of 37 years. The presence of larger metaplastic polyps was noted, and the possibility that “metaplastic polyposis” was a pathological entity was raised [29]. With follow-up, however, no cases of CRC were observed, thereby designating serrated polyposis as a low-risk condition of young males. This paper influenced thinking on serrated polyposis for over a decade. However, during the 1990s association with CRC was revisited by T orlakovic and Snover [7], Burt and Samowitz [38], and Jeeraratnam and colleagues in a familial setting [39] and in a small series of patients who were instrumental in demonstrating the malignant transformation of serrated polyps [8]. Many series and case reports have now been published [5–8, 19–21, 23, 24, 26, 28, 39–60]. In the three largest series published to date, 25–38% of patients presented with at least one CRC [9, 12, 27], and multiple CRCs were common [11]. However, the problem remains that published series are predominantly composed of retrospective clinic-based records, and therefore the estimation of CRC risk is likely to be inflated and to reflect a CRC risk associated with symptomatic patients. In addition, the wide variety of phenotypic presentations within serrated polyposis has the potential to be associated with varying risk magnitudes. At the time of writing, no prospectively collected population-based risk estimates for CRC are available. A summary of the findings in published series of serrated polyposis cases is presented in Table 1.

4. Serrated Polyp Subtypes

Serrated polyps are the second most common type of colorectal polyps, after conventional adenomas, found during population colonoscopy. Serrated polyps are also the most prominent phenotypic feature in serrated polyposis usually ranging in number from 5 to greater than 150, and varying greatly in size. In addition, a diverse range of dysplastic lesions of both conventional and serrated lineages may also be present.

In a study reporting the prevalence of each polyp type diagnosed from 179,111 consecutive population colonoscopies in the United States, Lash and colleagues found that epithelial benign polyps were classified as conventional adenomas in 58.6% and as serrated polyps in 41.4% [62]. The terminology and histologic classification of serrated polyps have been a matter of debate for some years. The most clinically relevant feature is the presence of dysplasia that increases the risk of developing CRC and impacts colonoscopy surveillance intervals. Therefore, the classification of serrated polyps into dysplastic polyps and nondysplastic polyps is the most meaningful division. However, there is still some degree of confusion in diagnosing serrated polyps with reported significant variation in the detection rate and in the histologic classification, justifying the need for increased awareness and education [63]. All of the following subtypes are observed in serrated polyposis.
Table 1: Summary findings in serrated polyposis from publications reporting more than five cases (adapted from Buchanan et al., 2010) [11].

<table>
<thead>
<tr>
<th>Author et al.</th>
<th>Year</th>
<th>Cases ($n$)</th>
<th>Mean age at diagnosis (years)</th>
<th>Number of polyps observed</th>
<th>% with CRC</th>
<th>Family history of CRC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kalady et al.</td>
<td>2011</td>
<td>115</td>
<td>62</td>
<td>2-multiple</td>
<td>25%</td>
<td>38%</td>
</tr>
<tr>
<td>Buchanan et al.</td>
<td>2010</td>
<td>126</td>
<td>49</td>
<td>5–150</td>
<td>40%</td>
<td>59%*</td>
</tr>
<tr>
<td>Boparai et al.</td>
<td>2009</td>
<td>77</td>
<td>56</td>
<td>2–53</td>
<td>35%</td>
<td>NS</td>
</tr>
<tr>
<td>Carvajal-Carmona et al.</td>
<td>2007</td>
<td>32</td>
<td>46</td>
<td>11-multiple</td>
<td>25%</td>
<td>59%</td>
</tr>
<tr>
<td>Chow et al.</td>
<td>2006</td>
<td>38</td>
<td>44</td>
<td>20-multiple</td>
<td>26%</td>
<td>50%</td>
</tr>
<tr>
<td>Renaud et al.</td>
<td>2001</td>
<td>28</td>
<td>58</td>
<td>20-multiple</td>
<td>29%</td>
<td>39%</td>
</tr>
<tr>
<td>Yeoman et al.</td>
<td>2007</td>
<td>24</td>
<td>61</td>
<td>5-multiple</td>
<td>54%</td>
<td>17%</td>
</tr>
<tr>
<td>Ferrández et al.</td>
<td>2004</td>
<td>15</td>
<td>53</td>
<td>15-multiple</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Lage et al.</td>
<td>2004</td>
<td>14</td>
<td>58</td>
<td>19–100</td>
<td>43%</td>
<td>33%</td>
</tr>
<tr>
<td>Hyman et al.</td>
<td>2004</td>
<td>13</td>
<td>62</td>
<td>20-multiple</td>
<td>54%</td>
<td>38%</td>
</tr>
<tr>
<td>Rashid et al.</td>
<td>2000</td>
<td>15</td>
<td>58</td>
<td>multiple</td>
<td>77%</td>
<td>38%</td>
</tr>
<tr>
<td>Leggett et al.</td>
<td>2001</td>
<td>12</td>
<td>57</td>
<td>30-&gt;100</td>
<td>58%</td>
<td>17%</td>
</tr>
<tr>
<td>Rubio et al.</td>
<td>2006</td>
<td>10</td>
<td>61</td>
<td>6–159</td>
<td>70%</td>
<td>10%</td>
</tr>
<tr>
<td>Spjut and Estrada</td>
<td>1977</td>
<td>9</td>
<td>53</td>
<td>Multiple</td>
<td>11%</td>
<td>NS</td>
</tr>
<tr>
<td>Williams et al.</td>
<td>1980</td>
<td>7</td>
<td>37</td>
<td>50–150</td>
<td>0%</td>
<td>14%</td>
</tr>
<tr>
<td>Torklakovic and Snover</td>
<td>1996</td>
<td>6</td>
<td>57</td>
<td>50–100</td>
<td>67%</td>
<td>NS</td>
</tr>
<tr>
<td>Place and Simmang</td>
<td>1999</td>
<td>6</td>
<td>60</td>
<td>50–100</td>
<td>50%</td>
<td>14%</td>
</tr>
</tbody>
</table>

NS: not specified or unknown.
*Genetics clinic series.

4.1. Nondysplastic Serrated Polyps. Nondysplastic serrated polyps comprise hyperplastic polyps and sessile serrated adenomas/polyps, representing the vast majority of all serrated polyps. The use of new high-definition endoscopes in association with chromoendoscopy or narrow-band imaging has led to a higher detection of these polyps.

4.1.1. Hyperplastic Polyps (HPs). More than 75% of serrated polyps are HPs [62, 64, 65]. Most often measuring 5 mm or less, HPs are sessile pale lesions, usually found on the tip of mucosal folds in the distal colorectum, with normal architecture and normal proliferation characteristics. In the proximal colon, HPs are often larger and more difficult to visualize. The prevalence of HPs in asymptomatic adults aged 40 years or more has been estimated to be around 10% in Western populations [66–68]. In autopsy studies, the prevalence rate of HPs ranged from 5% in a Cretan study to 40% in a British study [69–71]. While HPs develop at an earlier age than conventional adenomas, its incidence does not seem to significantly increase after 50 years, contrasting with the positive correlation between increased age and the prevalence of conventional adenoma [72].

Histologically, HP is the prototypical example of serrated polyps of the colon with a saw-toothed appearance caused by in-foldings of the crypt lining epithelium in the upper half of the crypts. All types of HP are characterized by elongated crypts, with maturation of cells towards the surface, and proliferation activity limited to the lower portion of the crypts. HPs are further divided into 3 histologic subtypes: microvesicular, goblet-cell, and mucin-poor, without clinical relevance as yet. Microvesicular HP is the most common type, characterized by the presence of columnar cells with abundant apical vesicular mucin and by a decreased number of goblet cells. In contrast, goblet-cell HPs show elongated crypts with numerous goblet cells and minimal serration limited to the most upper portion of the crypts. These polyps may be overlooked and are often underdiagnosed and therefore may occur more frequently than reports suggest. Finally, mucin-poor HPs are very rare and display prominent serration, regenerative changes, and mucin-depleted columnar cells. It is still debated whether mucin-poor HPs are a true separated subtype or an irritated form of microvesicular HPs.

4.1.2. Sessile Serrated Adenomas/Polyps (SSA/P). First designated “serrated polyps with abnormal proliferation” by Torklakovic et al. in 2003, SSA/Ps comprise 15–20% of all serrated polyps [65]. SSA/Ps are flat or slightly elevated lesions most commonly found in the proximal colon and usually measuring more than 5 mm. Histologically, SSA/Ps differ from HPs by the presence of abnormal architectural features secondary to abnormal proliferation. Whereas the proliferative zone in HPs is located in the base of the crypts, it is usually on the sides of the crypts in SSA/Ps, leading
to asymmetrical growth in crypts with an inverted T shape or L shape. Other histologic features of SSA/Ps include the presence of mature goblet cells at the bases of the crypts, hyperr sorration at the base or throughout the crypts, and pseudoinvasion of the muscularis propria. Dysplasia is not present.

4.2. Dysplastic Serrated Polyps

4.2.1. Traditional Serrated Adenomas (TSAs). TSA is a relatively uncommon polyp, comprising up to 5% of serrated polyps in Western countries with a higher prevalence in Asia, particularly in Korea [73]. Compared to SSA/Ps, TSAs are protuberant polyps more frequently found in the left colon and in older individuals. The architecture of TSA is often more complex than villous or tubulovillous adenoma, with prominent serration and finger-like projections. The presence of ectopic crypt foci in TSAs, defined by the crypts with their base not seated at the level of the muscularis propria, is useful to distinguish them from SSA/P [74]. The neoplastic cells are characterized by an abundant eosinophilic cytoplasm and elongated pencillate nuclei. Dysplasia in TSA is usually mild, with a different appearance of the dysplasia associated with conventional adenoma and low proliferative characteristics. However, conventional adenoma-type dysplasia can also be present and sometimes with high-grade features.

4.2.2. Sessile Serrated Adenomas/Polyps with Dysplasia (SSA/P-D). The presence of dysplastic crypts in a SSA/P was often reported as part of the “mixed polyp” group and is now better recognized as a specific category of dysplastic serrated polyps with malignant potential. In most cases, dysplasia in SSA/P is similar to dysplasia in conventional adenoma and is well demarcated from the nondysplastic areas. Dysplasia in SSA/Ps is rare, found in 14% of all SSA/P in a recent study by Lash et al. [62]. In this study of over 2000 patients, the median age for presenting with a nondysplastic SSA/P was 61, increasing to 66 for SSA/P with low-grade dysplasia, 72 for SSA/P with high-grade dysplasia, and 76 for SSA/P with invasive carcinoma (a span of 15 years). In contrast, the span between tubular adenoma and non-SSA/P carcinoma is only 5 years. Examples of serrated polyp subtypes are given in Figure 2.

5. Phenotypic and Molecular Heterogeneity

Table 2 shows the rates of molecular alterations for the major serrated polyp subtype categories, after adding all available results from various large studies [73, 75–84]. Somatic molecular alterations associated with serrated polyps have been well described in previous publications [54, 76, 79, 85–87] and include BRAF (V600E) mutation, KRAS (codons 12 and 13) mutations, MLH1 methylation, MGMT methylation, and CpG island methylator phenotype (CIMP). The prevalence of these alterations varies according to the subtype of serrated polyp. BRAF mutation is the most common alteration in all polyp types, with the highest rate in SSA/Ps (83.9%) and the lowest rate in goblet-cell HPs (20%). In contrast, KRAS mutation is most commonly detected in goblet-cell HPs (48.4%) and TSA (22.4%). MLH1 methylation ranges from 14.3% in goblet-cell HPs to 47.5% in TSA. Interestingly, while MLH1 methylation does occur quite frequently, a high-level microsatellite instability phenotype is very rarely encountered in serrated polyps [76, 88]. MGMT methylation ranges from 0% in goblet-cell HPs to 74.2% in TSA. A high level of CIMP, defined by ≥2/5 methylated markers, occurs in 39% of HPs, 76% of SSA/Ps, and 79% of TSA [76].

The question of phenotypic heterogeneity in serrated polyposis can be further considered with an examination of the reported molecular changes of serrated polyps as they apply to the syndromic patient. Though there appear to be at least two phenotypic subtypes which correspond to the first and last criteria (Text Box 2), a previous report from the UK clearly demonstrates that the predominant molecular change in the polyps is that of BRAF mutation, even in patients with numerous polyps [25], and therefore suggesting that oncogene mutations are of little value in subtyping this disorder. However, rare cases of serrated polyposis are reported where KRAS mutations predominate [25], and current evidence suggests that, in at least some of these patients, biallelic germline mutation of MUTYH may be responsible [25, 26, 90, 91].

Although the possibility of these two types of serrated polyposis was first raised over 10 years ago [92], the application of such a classification to CRC risk may not be readily implemented. Even though large and dysplastic polyps are likely to be an indicator of high malignant potential, the presence of CRC in cases with multiple small HPs [61], as well as the absence of CRC in many cases of serrated polyposis [11, 12] with large and dysplastic polyps, argues against a nonoverlapping classification.

6. Epigenetic Field Defect in Establishment of Neoplasia

CRC in general develops through one of two independent molecular pathways that involve sequences of genomic and epigenomic alterations associated with pathological and clinical features: the adenoma pathway in 70–80% and the serrated pathway in the remaining 20–30%. The somatic molecular features which characterize the serrated pathway to CRC include activating mutations in BRAF [81, 83, 86] and widespread hypermethylation of gene promoters (CIMP) [87] with or without MSI [8, 42]. In the serrated pathway, the earliest known event is somatic BRAF mutation, found in aberrant crypt foci [93], and with a high rate in microvesicular HP and SSA/P [76, 85]. The hypermature cells of the upper crypt in serrated polyps are thought to result from a mechanism of oncogene-induced senescence brought about by the presence of an activating BRAF mutation [94]. Escape from senescence is achieved subsequently
Figure 2: Showing 4 serrated polyp subtypes. (a) Microvesicular hyperplastic polyp with crypt serration and proliferative crypt bases. (b) Sessile serrated adenoma/polyp, showing asymmetrical serrated crypts and dilated crypts. (c) Traditional serrated adenoma, with prominent complex serration and hypereosinophilic cells. (d) Sessile serrated adenoma/polyp with high-grade dysplasia (right).

Table 2: Prevalence of genetic and epigenetic alteration in the different subtypes of serrated polyps by combining all comparable data from [73, 75–84].

<table>
<thead>
<tr>
<th>Polyp type</th>
<th>BRAF mutation</th>
<th>KRAS mutation</th>
<th>CIMP-H*</th>
<th>MLH1 methylation</th>
<th>MGMT methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microvesicular</td>
<td>66.3%</td>
<td>12.3%</td>
<td>47.4%</td>
<td>39.5%</td>
<td>26.3%</td>
</tr>
<tr>
<td>Goblet-cell</td>
<td>20%</td>
<td>48.4%</td>
<td>14.3%</td>
<td>14.3%</td>
<td>0%</td>
</tr>
<tr>
<td>All subtypes</td>
<td>51.5%</td>
<td>22.1%</td>
<td>38.5%</td>
<td>27%</td>
<td>14.8%</td>
</tr>
<tr>
<td>SSA/P</td>
<td>83.9%</td>
<td>5.8%</td>
<td>75.9%</td>
<td>42.6%</td>
<td>25%</td>
</tr>
<tr>
<td>TSA</td>
<td>62.5%</td>
<td>22.4%</td>
<td>79.3%</td>
<td>47.5%</td>
<td>74.2%</td>
</tr>
</tbody>
</table>

*CIMP-H: high level of CIMP in polyps defined by ≥2/5 methylated markers from O’Brien et al. [76, 85].

by the inactivation by promoter methylation of tumour suppressors controlling senescence [95], thus allowing a lesion to progress to a more proliferative neoplasm. An epigenetic field defect present in serrated polyposis would facilitate this process more readily [30] with the consequent increase in polyp numbers which define the condition. When multiple lesions are examined, serrated pathway features of BRAF mutation and CIMP demonstrate a high rate of concordance between discrete lesions in individuals with serrated polyposis [86, 87]. As would be expected, most CRCs (70%) in serrated polyposis derive from the proximal colon [17]. An exception to this occurs in young-onset patients (<50 yrs old) where the CRCs are more likely to be distal [11, 12, 26]. This is an under-recognized feature of young-onset serrated polyposis though it has been mentioned in previous publications [96].

Hypermethylation of gene promoters is also observed in the normal mucosa of individuals with increasing age [97, 98] and is also more likely to be associated with synchronous proximal CRC with concordant molecular features [99, 100]. However, in serrated polyposis, increased methylation of gene promoters is evident even in the normal mucosa of younger individuals [87, 91, 101], indicating that an epigenetic regulatory defect may be present in the normal tissues of these patients and suggesting a prematurely aged mucosa associated with increased risk for the establishment of neoplasia. Of interest, in 1968, Arthur observed that metaplastic polyps were a marker of age in the normal mucosa
The concept of an epigenetic field defect in serrated polyposis was clearly demonstrated by Minoo et al. in 2006 [30]. Significantly the level of methylation in apparently normal mucosa was higher in serrated polyposis patients when compared to patients with sporadic serrated polyps.

7. Role of the Conventional Adenoma in Cancer Risk

The complex biology of serrated neoplasia and the plasticity of its developmental pathway can give rise to CRC with variable MSI status [8], and to small numbers of apparently conventional adenomas, in addition to multiple serrated polyps. It has been estimated that conventional adenomas are seen in up to 90% of serrated polyposis patients [23, 24], raising the notion that lesser numbers of conventional adenomas are part of the syndrome. Importantly, the risk for patients with serrated polyposis to present with a synchronous CRC at time of diagnosis is significantly higher when at least one conventional adenoma is present [11, 12, 24].

Adenomatous lesions in a serrated polyposis patient may either evolve from serrated polyps, progressing to a conventional type of dysplasia, or arise via an alternate mechanism. The presence of “mixed polyps,” as they were previously known (now called SSA/P with dysplasia), which demonstrate a very high rate of somatic BRAF mutation (80–90%) [76] and therefore, by implication, origin in a serrated polyp, provides a plausible precursor lesion for the CRC which arises in serrated polyposis [6, 19, 50, 56, 57]. In contrast, conventional adenomas almost never harbor a BRAF mutation [84]. If most of the CRCs in serrated polyposis were to arise from advanced serrated polyps, a high rate of BRAF-mutated CRC would be expected. CRCs in serrated polyposis have shown somatic BRAF mutation in 33% of 6 cases in an early published report [86] and, consistently, in 19/58 (33%) of a recent case series, [102]. Whilst this level is still greater than that of a population series, (which ranges from 7% in southern Europeans to 21% in Anglo-Celts [103]), these observations suggest that the majority of CRCs arising in serrated polyposis develop within lesions not known to be involved in the canonical serrated pathway (see Figure 3).

The balance of CRC (BRAF wild-type) in serrated polyposis either demonstrates somatic KRAS mutation at a rate of approximately 19% which is half that of the population [102] or is oncogene mutation null. These CRCs may arise, in the manner of common CRC, from the conventional adenomas which frequently coexist in patients with serrated polyposis [17, 23]. Of interest, in reporting the first autosomal dominant family with serrated neoplasia, Jeevaratnam et al. made note of an adenomatous precursor in contiguity with a small CRC [39] and concluded that the CRCs in serrated polyposis arise through both the development of dysplasia in serrated polyps and through coincident conventional adenomas. In 2010, Pai et al. published a report which examined the adenomas which coexisted with SSA/P in the general (non-syndromic) population [105]. They observed that 35% of the polyps removed from population patients with an index SSA/P were conventional adenomas. Pai et al. observed 3 morphologic features which were more prevalent in the study adenomas compared to control adenomas in a population without SSA/P: eosinophilic cytoplasm, focal (rather than widespread) serration, and crypt dilatation. These features were seen in 30% of the study adenomas compared to 2.5% of controls. In addition, these atypical polyps demonstrated low levels of methylation, and increased staining for MUC6, properties more associated with serrated lesions than with adenomas. Importantly, none of these lesions showed BRAF mutation. Given previous findings from the same group of authors which demonstrated that patients with an index SSA/P had a significantly increased risk of having other serrated polyps, combined with the presence of atypical conventional adenomas, supports the presence of an epigenetic field defect in serrated polyp patients including those not meeting the criteria for serrated polyposis [105, 106].

Small numbers of serrated polyps as well as CRCs with somatic KRAS mutation are also observed in serrated polyposis patients. Somatic KRAS mutation straddles the division between serrated and adenomatous polyps and is mutually excluded in lesions bearing BRAF mutation [107]. It is observed in goblet-cell HPs [85] which rarely undergo malignant transformation; however, its presence, albeit less frequently, in advanced serrated polyps [84], in rare contiguous serrated polyps attached to population CRC (Young, unpublished observations) and in the lesions present in biallelic mutation carriers for MUTYH [90], suggests that serrated lesions with KRAS mutations are not completely devoid of malignant potential.

8. The Smoking Paradox and Serrated Polyposis

Risk factors for the development of serrated polyps in the population are similar to risk factors for the development of conventional adenomas, including alcohol consumption, low folate intake, and high body mass index [72, 108]. High calcium intake, hormone replacement therapy, and use of nonsteroidal anti-inflammatory drugs are associated with a reduced risk. Perhaps the most interesting of risk factor associations involves cigarette smoking. The relationship between smoking and colorectal neoplasia has become known as the smoking paradox in that smoking is associated significantly with polyps, but its relationship with CRC is much weaker. A consistent observation has been the relationship between smoking and serrated polyps, which has been analyzed in a number of population-based studies [109]. Three independent studies have demonstrated a concordant pattern of higher risk estimates for serrated (hyperplastic) polyps than for conventional adenomas [110–112] in long-term and current smokers. When both serrated polyps and conventional adenomas were present [110–112], risks were higher still. The association of current smoking with serrated polyps begins very early in serrated neoplasia [113] and is the greatest in the distal colon [114], where malignant potential of the serrated polyps is low. The weak association between smoking and CRC has been explained by the relationship being dominated by the serrated pathway subset with BRAF mutation [115], which accounts for only 15% of CRC.
Originally the canonical serrated pathway was devised to explain CRC with two highly associated molecular markers, \textit{BRAF} mutation and CIMP. \textit{BRAF} mutation is an early contrast serrated polyps with \textit{BRAF} mutation in the distal colon rarely progress to CRC. Increasing CIMP in progression to CRC is shown as a pale blue arrow.\n
\begin{itemize}
\item \textit{BRAF} 83\%\hspace{2cm} Only 2\% in distal colon
\item \textit{BRAF} 57\%\hspace{2cm} Only 12\% in distal colon
\end{itemize}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{serrated_pathway.png}
\caption{The canonical serrated pathway showing progression through MVHP (microvesicular hyperplastic polyp) to SSA/P (sessile serrated adenoma/sessile serrated polyp) and SSA/P-D (SSA/P with dysplasia) to CRC with CIMP and high levels of \textit{BRAF} mutation frequently arising in the proximal colon. CRC with CIMP can evolve into MSI-H and non-MSI-H subtypes. Though KRAS mutation can be observed in CIMP CRC, these are relatively rare [103, 104].}
\end{figure}

The role of smoking in serrated polyposis has not been extensively explored. In 2010, several reports added to the puzzle surrounding the smoking paradox. Initially it was demonstrated that current smoking is associated with a significantly higher polyp count in patients with serrated polyposis [11]. Also in 2010, a report suggesting a causative role for smoking was published on a small series of cases and showed a higher prevalence of current smokers amongst serrated polyposis patients than in the population [116]. Given that current smoking is associated with increased serrated polyps [114] and even serrated aberrant crypt foci in the general population [113], both the preceding observations in serrated polyposis patients are reasonable.

Later in 2010, the authors of this current review reported that, even though current smoking was associated with increased polyp numbers, there was no significant effect on the risk of CRC in a case series of 151 patients with serrated polyposis [12], once again highlighting the smoking paradox. Given that the major association between smoking and CRC is largely confined to those CRCs with somatic \textit{BRAF} mutation, and less than one-third of CRCs in serrated polyposis harbor a \textit{BRAF} mutation, this is perhaps not unexpected, as \textit{BRAF}-mutated CRCs constitute a minority of serrated polyposis CRCs. Of interest, however, an unexpected finding emerged regarding currently smoking females, who had a significantly decreased risk of CRC, after correcting for age and adenomas [12] (O.R 0.10, 95\% CI 0.02 to 0.47, \( P = .004 \)). Further, female patients who had ever smoked had an average age of onset for CRC of 63 years compared to those who had never smoked (50 yrs). Though the results did not reach statistical significance due to low numbers, a trend for delayed onset of CRC in female smokers was evident. In the population, female smokers with serrated pathway subset CRC are elderly onset.

The preceding observation is consistent with a biological mechanism similar to that reported in ulcerative colitis patients [117] and suggests that perhaps an inflammatory process may be responsible for neoplastic progression in serrated polyposis in a subset of female patients and that, similarly to ulcerative colitis, smoking may be anti-inflammatory. In the population, a study of risk factors for serrated polyps demonstrated that aspirin use decreased the risk of advanced proximal polyps, lending indirect support to this hypothesis [114]. Alternatively, confounding factors may be responsible for this finding, including unspecified sex-specific factors related to body mass index (BMI) or hormonal factors, as observed in the protective effects of smoking on endometrial cancer. This finding whilst perplexing cannot be ignored because it could potentially lead to a CRC-preventive modality for female patients with serrated polyposis independent of cigarette smoking and its attendant harms.

\section{9. Serrated Polyposis as a Genetic Predisposition Syndrome}

Serrated polyposis has many hallmarks of a genetic predisposition. These include an earlier age of onset of CRC, polyp, and cancer multiplicity, increased CRC risk in both patients and their relatives, and restricted ethnicity. An important
clinical consequence associated with serrated polyposis is the increased risk of both CRC [26, 39, 43, 44, 54] and possibly extracolonic cancers [27, 118, 119] in the family setting of serrated polyposis patients. The risk to first-degree relatives of CRC has been estimated at fivefold greater than that of the general population [10]. WHO Criterion 2 (Text Box 2) addresses the evidence that serrated polyposis may occur in a familial context [25, 26, 40, 43, 48, 54, 61] and elevates the significance of smaller numbers of hyperplastic polyps in a first-degree relative of an individual with serrated polyposis. The genetic basis for serrated polyposis is yet to be determined, though small numbers of patients have reported mutations in MUTYH [90], PTEN [120], and EPHB2 [121].

Biallelic MUTYH mutation is a phenotypically diverse disorder which appears to interact with the genetic background of the individual. In one-third of biallelic mutation carriers, there are no adenomas present [122]. In approximately 1% of patients with serrated polyposis, biallelic mutation of MUTYH can be demonstrated [123]. Conversely, when biallelic MUTYH mutation carriers are assessed, 18% meet the WHO criteria for serrated polyposis [90]. A recent report from Buchanan et al. [124] suggests that individuals with both MUTYH-associated polyposis (MAP) and serrated polyposis may be segregating two conditions with diverse modes of inheritance. The report describes a 56-year-old Caucasian male with >100 colonic polyps (approximately 50 conventional 10–15 mm adenomas predominating in the proximal colon and approximately 50 < 5 mm serrated polyps in the distal colon and rectum) who also demonstrated biallelic mutation for the two common European variants in MUTYH [124]. His mother had CRC of the sigmoid colon at 70 yrs. His 17-year-old symptomatic son who was not a biallelic mutation carrier had multiple 4 mm hyperplastic polyps in the rectosigmoid. The implications of this case report are that the risk to first-degree relatives of biallelic MUTYH mutation carriers (RR < 1.5) [125] is not as substantial as in serrated polyposis where the risk is fivefold greater than the population risk [10]. Therefore screening protocols in the setting described need also to consider the extra risk to first-degree relatives that serrated polyposis can pose. For this reason, detailed pathological examination of the polyps in patients with MAP is recommended to exclude coexisting serrated polyposis. Should serrated polyposis also be present, screening beyond siblings should be considered.

The evidence that serrated polyposis is a genetic predisposition is accumulating. Though multiple cases of serrated polyposis within a single family are rare [39, 54], the phenotype of multiple neoplasms, young-onset, and occasional affected sibships including consanguineous kindreds [26] suggest a pattern of inheritance consistent with an autosomal recessive or codominant mode [13, 126]. Codominant modes of inheritance result in an intermediate phenotype when one variant risk allele is present and a significantly altered phenotype in those where both alleles are variants.

10. Surveillance and Cancer Prevention Approaches

Several reports have suggested that malignant transformation in the serrated pathway may be unusually rapid in some clinical settings. Despite these observations, the apparent rapid evolution to cancer of advanced serrated polyps remains unproven and may be due to the difficulty of visualizing flat serrated lesions at colonoscopy. SSA/P progress very slowly to dysplasia in a population cohort [62]; however, in the syndromic patient this progression may be more rapid in an epigenetically abnormal environment. Hyman and colleagues reported 3 cases of serrated polyposis where CRC developed despite colonoscopy every 2 years [43]. Similarly, Azimuddin and colleagues reported that colonoscopy every 3 years was inadequate for some families with atypical serrated polyps [40]. Boparai et al. reported that 5 of 77 serrated polyposis patients developed a CRC whilst under surveillance for 5 years [9]. In four of five, the CRC arose within 12 months of a previous colonoscopy; however, many polyps had been left in situ. Currently, the issue of rapid evolution in serrated polyposis remains unresolved.

There are no clear guidelines though recommendations are evolving [127]. Frequent surveillance colonoscopy in the initial period after diagnosis both to allow endoscopic control of the polyps and to determine the nature and progress of the disease appears justified. Appropriate subsequent surveillance intervals can then be determined, but to avoid interval CRC this is unlikely to extend beyond 2-3 year intervals. Referral to a tertiary centre should be planned particularly if the polyp burden is difficult to control endoscopically and surgery is being considered.

11. Summary and Future Directions

Serrated polyposis is a condition with an increased CRC risk to both individuals and their relatives. An understanding of the mechanism of malignant transformation in serrated polyposis is still evolving, along with the risk factors which influence it [96]. Without a known germline sequence variant and estimated genetic penetrance, the identification and management of individuals and their families with a CRC predisposition syndrome become increasingly problematical. The prospect of a syndrome with a codominant mode of inheritance presents particular difficulties in that, although some individuals will present with a florid phenotype, such as that seen in serrated polyposis, first-degree relatives may have only a few polyps or none at all. The role of genetics departments, pathologists, and endoscopists in understanding the clinical picture for such families is likely to become increasingly important and interdependent. The challenge will be to determine if we can confidently identify and assign CRC risks to the different phenotypes of serrated polyposis, thereby allowing tailored clinical management with regard to the frequency of colonoscopic surveillance, the aggressiveness of polyp removal, and consideration of colonic resection.
Conflict of Interests

The authors have no conflict of interests to declare with respect to this work.

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References


Review Article

CpG Island Methylation in Colorectal Cancer:
Past, Present and Future

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The concept of a CpG island methylator phenotype, or CIMP, quickly became the focus of several colorectal cancer studies describing its clinical and pathological features after its introduction in 1999 by Toyota and colleagues. Further characterization of CIMP in tumors lead to widespread acceptance of the concept, as expressed by Shen and Issa in their 2005 editorial, "CIMP, at last." Since that time, extensive research efforts have brought great insights into the epidemiology and prognosis of CIMP+ tumors and other epigenetic mechanisms underlying tumorigenesis. With the advances in technology and subsequent cataloging of the human methylome in cancer and normal tissue, new directions in research to understand CIMP and its role in complex biological systems yield hope for future epigenetically based diagnostics and treatments.

1. Introduction

In the October 29, 2010 issue, Science turned the spotlight on epigenetics—a term that encompasses histone modification, nucleosome location, noncoding RNA, and DNA methylation. Epigenetic processes do not involve changes to DNA sequence but rather are self-propagating molecular signatures that are potentially reversible, unlike changes in genetic information [1]. DNA methylation is the most widely studied epigenetic marker [2]. The discovery of global DNA hypomethylation in human tumors was followed by the identification of hypermethylated tumor-suppressor genes and recently, inactivation of microRNA (miRNA) by DNA methylation also has been described [3–5]. Growing interest in epigenetic systems stems from an inability to determine causative genetic variants in many disorders. It is hoped that a better understanding of these systems may provide insight into our understanding of complex diseases such as cancer.

Approximately half of all protein-encoding genes in the human genome contain CG-rich regions in their promoters or CpG islands. Aberrant DNA methylation, in the form of hypermethylation of CpG islands, results in repression of transcription in tumor suppressor genes. For example, inactivation of the mismatch repair gene MLH1 by promoter methylation is the molecular basis for microsatellite instability in sporadic microsatellite unstable colon cancers [6]. This phenomenon of tumor alteration via epigenetic silencing associated with dense hypermethylation of CpG islands, and their complex interplay with modifications in histone structure, provides an alternate mechanism to genetic inactivation of tumor suppressor genes via loss or mutation [2]. The presence of widespread CpG island methylation in a tumor is termed the CpG island methylator phenotype, or CIMP, and this paper is focused on this specific aspect of epigenetics.

2. CIMP in Colorectal Cancer

The role of CIMP in colorectal carcinogenesis was originally postulated in 1999 by Toyota et al. [7]. Their pioneering study distinguished between age-related and cancer-related methylation and defined CIMP in terms of the latter. Recognition of CIMP as a phenomenon in colorectal cancer is relatively recent; more than a decade earlier, in 1988, Vogelstein and colleagues developed a model that hypothesized that
colorectal neoplasia occurs from a series of genetic alterations that includes activation of oncogenes and inactivation of tumor suppressor genes [8]. The concept of an epigenetic that includes activation of oncogenes and inactivation of colorectal neoplasia occurs from a series of genetic alterations [6, 9, 10]. However, the existence of CIMP has since gained wide acceptance, as the epidemiology characterizing this epigenetic alteration and its utility in understanding carcinogenic pathways support its significance in colorectal cancer biology [11–14].

Most sporadic microsatellite unstable colon tumors are CIMP positive, whereas CIMP is uncommon in Lynch syndrome-associated cancers which exhibit microsatellite instability (MSI), indicative of distinct underlying molecular processes [13, 15]. Based on a number of relatively large case-control and prospective cohort studies, ~30–40% of sporadic proximal-site colon cancers are CIMP positive, compared to 3–12% of distal colon and rectal cancers [16–21] as illustrated in Figure 1. Thus CIMP is significantly more frequent in tumors of the proximal colon, and this is independent of MSI status. CIMP is also associated with BRAF mutations in both microsatellite stable and unstable colon cancers [11, 18, 20]. CIMP is observed in proximal hyperplastic (serrated) polyps, suggesting this lesion may be a precursor to unstable tumors (and perhaps stable tumors) in the CIMP high pathway [22]. Once thought to lack potential for malignant progression, hyperplastic polyps are now considered to represent a heterogeneous group, most of which harbor BRAF mutations and some of which exhibit epigenetic alterations (both uncommon in colorectal adenomas). A subset of hyperplastic polyps has been defined by architectural features and renamed sessile serrated polyps (or sessile serrated sessile adenomas). Most of these polyps are right-sided and many show CIMP, supporting the notion that they may be a precursor lesion for CIMP high tumors [23, 24].

Some case-control and cohort studies have reported a poor prognosis associated with CIMP in combination with microsatellite stable tumors [19, 25–27], although this may reflect the co-occurrence of BRAF V600E mutations, which have been associated with significantly poorer survival in colon cancer [28, 29]. Relatively minor effects of CIMP on prognosis suggest that the effect of mutations in BRAF on survival in stable tumors is not dependent on CIMP [16, 28]. Indeed, Ogino et al. reported that CIMP-high appears to be an independent predictor of a low colon cancer-specific mortality [30]. These results suggest the need for a large sample size to determine the relative contributions of BRAF and CIMP on prognosis.

### 3. Characterization of CIMP

In contrast to the relatively straightforward determination of MSI tumor status, a consensus as to the optimal panel of CpG sites for CIMP determination is only starting to take shape (Table 1). Different panels may yield slightly different results, although a strong relationship to the presence of a BRAF V600E mutation is consistently observed with all panels. The so-called “classic” panel of Park et al. utilized to assess CIMP status consists of CpG sites in MLH1, CDKN2A (p16), and methylated in tumors (MINTS) 1, 2, and 31 [31]. It has been suggested that there are two general types of CIMP in sporadic tumors: CIMP high, related to BRAF mutations and MLH1 methylation; and CIMP low, related to KRAS mutations [12, 32, 33]. Tumors characterized as CIMP positive (CIMP+) based on the classic panel include both CIMP high and CIMP low categories; therefore, a subset of CIMP+ associates with BRAF and another with KRAS mutations, somewhat surprising given mutations in these genes are typically mutually exclusive since both are members of the ras signal transduction pathway [6, 11].

![Figure 1](image)

**Figure 1:** In colorectal cancer, CIMP+ occurs more frequently in tumors of the proximal colon (Figure 1(a)) and less frequently in tumors of the distal colon and rectum (Figure 1(b)). An approximate distribution of genetic and epigenetic tumor alterations is shown.

Using structural equation modeling to construct causal models of CIMP and locus-specific CpG island methylation and a large database of colorectal cancers, Tanaka et al. showed the correlation structures of 16 methylation markers and CIMP status differed between BRAF mutated, KRAS mutated, and wildtype BRAF/KRAS tumors [35]. They suggested a possible role of these mutations differentially modifying the propensity for locus-specific methylation at the cellular level. To examine the question of whether or not BRAF V600E plays a causal role in the development of


**Table 1: A history of CIMP panels used to assess CpG island methylation in colorectal cancer.**

<table>
<thead>
<tr>
<th>Study</th>
<th>CIMP panel markers</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toyota et al. [7]</td>
<td>CDKN2A (p16), MINT1, MINT2, MINT12, MINT17, MINT25, MINT27, MINT31, MLH1, THBS1</td>
<td>Pioneering work to identify markers that distinguish CIMP from age-related methylation</td>
</tr>
<tr>
<td>Park et al. [31]</td>
<td>CDKN2A, MINT1, MINT2, MINT31, MLH1</td>
<td>So-called “classic” or traditional panel</td>
</tr>
<tr>
<td>Weisenberger et al. [13]</td>
<td>CACNA1G, IGF2, NEUROG1, RUNX3, SOCS1</td>
<td>“New” panel based on stepwise screen of 195 markers</td>
</tr>
<tr>
<td>Ogino et al. [34]</td>
<td>CACNA1G, CDKN2A, CRABP1, MLH1, NEUROG1</td>
<td>Selected markers to distinguish high-level from low-level methylation</td>
</tr>
<tr>
<td>Shen et al. [35]</td>
<td>CIMP1: MINT1, MLH1, RIZ1, TIMP3, BRAF mutation; CIMP2: MINT2, MINT27, MINT31,</td>
<td>Examined 27 CpG sites, proposed optimal epigenetic and genetic markers to identify CIMP1, CIMP2, or CIMP-</td>
</tr>
<tr>
<td></td>
<td>Megalin, KRAS mutation</td>
<td></td>
</tr>
<tr>
<td>Tanaka et al. [35]</td>
<td>CACNA1G, CDKN2A, CHFR, CRABP1, HIC1, IGF2, IGFBP3, MGMT, MINT1, MINT31, NEUROG1,</td>
<td>Correlation structures of markers and CIMP differ by KRAS and BRAF status</td>
</tr>
<tr>
<td></td>
<td>p14, RUNX3, SOCS1, WRN</td>
<td></td>
</tr>
<tr>
<td>Ang et al. [36]</td>
<td>Total of 202 CpG sites differentially methylated between tumor and normal</td>
<td>Comprehensive DNA methylation profiling in 807 cancer genes</td>
</tr>
<tr>
<td>Kaneda and Yagi [37]</td>
<td>Group 1: IGF2, LOX, MINT1, MINT2, MINT31, MLH1, RUNX3, SOCS1; Group 2: ADAMTS1,</td>
<td>Comprehensive DNA epigenotyping of genomewide regions indentified two groups (high and intermediate to low methylation)</td>
</tr>
<tr>
<td></td>
<td>DUSP26, EDIL3, ELMO1, FB2, HAND1, IGFBP3, NEUROG1, RASSF2, STOX2, THBD, UCHL1</td>
<td></td>
</tr>
</tbody>
</table>

CIMP, Hinoue et al. determined 100 CIMP-associated CpG sites and examined changes in DNA methylation in eight stably transfected clones over multiple passages [38]. They observed that BRAF was not sufficient to induce CIMP in their system.

In contrast to evaluation of relatively small sets of CIMP markers, comprehensive DNA methylation profiling and unsupervised hierarchical clustering were recently used to identify several CpG sites that were differentially methylated between tumor and normal tissue [36]. Using a similar approach, the use of two methylation panels as classifiers of colorectal cancer has been proposed: the first to identify highly methylated tumors (strongly correlated with BRAF) and a second to distinguish between intermediate (associated with KRAS) and low methylation groups [37]. Since epigenetic therapy is in clinical use or trials for several cancers, efficient methods for epigenetic profiling are needed; Kondo and Issa provide a summary of available profiling techniques and their features [39]. As approaches to CIMP characterization in colorectal cancer continue to evolve, it is clear that BRAF and KRAS oncogene mutations will continue to refine any definition of CIMP. Although characterization of CIMP status depends on methylation markers and criteria used, classification of tumors by both CIMP and MSI status recently proposed by Jass [40] and further refined by Ogino and Goel [14] has become an increasingly common strategy to define the pathological and clinical features of colorectal cancer.

## 4. Epidemiology of CIMP

### 4.1. Characteristics

Relationships between CIMP and clinicopathologic features of colorectal tumors that have been widely reported include proximal location, older age at diagnosis, female gender, poor tumor differentiation, MSI (CIMP high cancer), BRAF mutations, KRAS mutations (microsatellite stable cancer), and wildtype TP53 [11, 13, 16, 17, 34, 41]. Based on a large Australian cohort, English et al. reported that individuals of southern European ethnicity had lower risk of CIMP and BRAF mutation than those with origins in northern Europe [21].

Using actual data and a classification tree method to visualize carcinogenic pathways, Slattery et al. suggested that unique mutational pathways to colon and rectal cancer likely exist [18]. This method describes the probability of developing various alterations in proximal colon, distal colon, or rectal tumors given previously acquired mutations. Using bootstrap resampling, the probabilities of developing specific mutations differed across tumor sites. For example, the estimated proportion of tumors that will develop methylation at CpG sites decreased as one goes from proximal colon to rectal cancers. Regardless of site, a methylation pathway in which BRAF is subsequently acquired independent of MSI or MLH1 methylation was predicted. This work supports previous observations that link CIMP and BRAF mutation, independent of MSI status [11, 12].

### 4.2. Smoking

The presence of methylation in human malignancies bears a relationship to a history of cigarette smoking. Cigarette smoking has been associated with CpG island methylation within the bronchial epithelium of smokers and in lung cancer, and activation of the aromatic hydrocarbon receptor by cigarette smoke has been associated with CpG methylation [42–44]. A significant relationship has been reported between cigarette smoking and CIMP (and closely related mutations in BRAF) in colon and rectal carcinomas in both prospective cohort and case-control studies [45–48]. Interestingly, the relationship between smoking and CIMP
4.3. Other Risk Factors. S Adenosylmethionine (SAM), the universal donor of methyl groups in humans, and S Adenosylhomocysteine (SAH), the product of and an inhibitor of DNA methyltransferase (DNMT) enzymes, provide connections between folate metabolism and DNA methylation [51]. It has been hypothesized that diets low in folate and high in alcohol intake may disturb DNA methylation, which may result in global DNA hypomethylation concurrently with a greater risk of cancers with CpG island methylation [52, 53]. In contrast, other studies have shown that global DNA hypomethylation is inversely correlated with CIMP and may represent different pathways to colorectal cancer [54, 55]. Studies generally do not support a unique role for alcohol and folate in CIMP+ tumors [56, 57], although genetic polymorphisms in MTHFR 1298A > C (rs1801131), interacting with diet, and TCN2 776G > C (rs1801198) may be involved in the development of highly CpG-methylated colon and rectal cancers [58–60]. Conversely, MTHFR 1298A > C was not associated with CIMP+ tumors in the Netherlands cohort study [61]. Polymorphisms in DNA repair genes have been implicated in CIMP-positive colon cancer [62, 63]. A promoter polymorphism in MLH1 (−93G > A) was associated with CIMP, MLH1 methylation, and BRAF mutations in unstable sporadic colon tumors and not in stable tumors, suggesting the genetic variant may be acting at a relatively late stage of carcinogenesis to drive CIMP-positive tumors down the microsatellite instability pathway [63].

Overexpression of DNMT3B has been shown to be a risk factor for the development of CIMP in colorectal cancer [64, 65]. DNMT3B is important in establishing and maintaining genomic methylation patterns, and overexpression in mice can induce tumors with methylation in specific CpG islands. Recent findings indicate that DNA methylation changes occur sequentially during tumor progression, and DNMT3B expression levels increase during this progression [66].

The future of CIMP in colorectal cancer research may well take place in the evolving trans- and interdisciplinary field of “molecular pathological epidemiology” outlined by Ogino et al, which is designed to elucidate how genetic factors and lifestyle exposures interact with specific molecular subtypes of cancer [67]. Hughes et al. reported that severe caloric restriction was associated with decreased risk of developing a tumor characterized by CIMP. This study provides a potential link between early life conditions and epigenetic changes that later influence colorectal cancer development [68]. The work of Slattery et al. regarding differences in the etiologies of rectal-site and colon-site tumors, and the influence of genetic factors in the inflammatory pathway in the etiology of CIMP in both, is an example of this approach [69, 70].

5. Emerging Trends in CIMP Research

Although aberrant DNA methylation of promoter CpG islands in cancer genes as well as repressive chromatin are frequently involved in gene inactivation during tumorigenesis, the mechanisms underlying CIMP are poorly understood. Patterns of hypermethylation are specific to tumor type, and it is unclear why certain regions become hypermethylated; however, mapping of the human methylome as a result of technological advances has expanded our understanding of epigenetic mechanisms [71]. Inactivation of particular genes may confer a growth advantage, resulting in clonal selection [72]. Another possibility is that long-range epigenetic silencing by DNA methylation can span chromosome regions of 1 Mb in colorectal cancer, resembling the loss of heterozygosity often observed in tumors [73]. In a large cohort of sporadic colorectal cancers, Wong et al. reported a strong relationship between long-range silencing of chromosome region 3p22 and CIMP+ tumors [74].

Recent findings suggest that most DNA methylation alterations in colon cancer occur in CpG island shores, sequences up to 2 kb distant from CpG islands [75]. Hypomethylated CpG shores appear closer to their associated CpG islands, while hypomethylated shores occur further away from their associated islands and resemble noncolon normal tissues. These findings are consistent with an epigenetic progenitor model of cancer, in which epigenetic alterations affecting tissue-specific differentiation are the predominant mechanism by which epigenetic changes cause cancer. Alternative transcription may be a function of differential DNA methylation during differentiation, and one role of altered methylation in cancer may be to disrupt regulatory control of specific promoter usage [75].

Previous studies suggest a general model in which genes reposition away from the heterochromatin when activated and gravitate to heterochromatin when silenced [76]. However, Easwaran et al. demonstrated that aberrant silencing of cancer-related genes occurred without requirement for their being positioned at heterochromatic domains using immunostaining for active/repressive chromatin marks and fluorescence in-situ hybridization in CRC cell lines. Furthermore, CpG hypermethylation, even associated with long-range silencing of nearby genes, occurred independently of their heterochromatic or euchromatic location [77]. These findings have important implications for understanding relationships between gene expression patterns and nuclear organization in cancer.

Another area under investigation is the understanding of mechanisms underlying which tumor suppressor genes are targeted for inactivation in cancer. Studies suggest a stem cell origin linked to epigenetic control of gene expression patterns in precursor cells regulated by constituent proteins in PcG repressive complexes including Polycomb Repressive Complex PRC1 [78, 79]. It was subsequently shown that sustained expression of the PRC1 protein, CBX7 along with
of active assessment of methylation in CIMP-specific promoters from tumors and other specimens. For example, quantitative assessment of methylation in CIMP-specific promoters of MLH1, WRN, and other DNA-repair genes in colon tumors, in comparison to normal tissues, may predict response to treatment [2]. Profiles of miRNA expression also differ between tumor and normal tissues; silencing of miR-124a in colon cancer cells activates expression of the oncogene CDK6 [5]. Continued research involving detailed DNA methylomes in healthy and diseased tissues will help distinguish causal epigenetic alterations from “bystander changes” which are a consequence of cellular processes [71].

Unlike mutations in DNA sequence, epigenetic alterations such as CpG Island hypermethylation are potentially reversible by “reawakening” silenced tumor suppressor genes. Two nucleoside DNA methylation inhibitors, azacitidine and decitabine, are used clinically in low doses to treat myelodysplastic syndrome, providing proof of principal for epigenetic therapy [81]. Structurally, these agents mimic cytosine; during cell replication, false cytosines replace real cytosines in growing DNA strands and then trap DNA methyltransferases to interfere with the ability of these enzymes to reproduce existing methylation in new cells. While inhibiting DNA methylation is a targeted molecular approach to therapy, downstream effects on neoplastic behavior are nonspecific and may result in cytotoxic cell death, making predictions of clinical outcomes difficult [81]. Clinical trials are being extended to test DNMT inhibitors in solid tumors of the breast, lung, and colon, in combination with histone deacetylase (HDAC) inhibitors which provide synergistic benefits in cell studies [82]. Other treatment avenues on the horizon include induced cellular programming to guide development of epigenetic-modifying drugs [83].

It has been a little over a decade since the concept of a CpG island methylator phenotype in colorectal cancer was introduced, and subsequent focus of several studies on describing the clinical and pathological features of CIMP as well as its characterization in tumors has supported widespread acceptance of the role of DNA methylation in cancer (Figure 2). The past few years have brought substantial insights as to the mechanisms underlying the CIMP pathway in cancer, and the future development of diagnostics and treatments based on our understanding of this epigenetic alteration are an exciting development in epigenetic research.

References


Review Article

Molecular Pathology of Hepatic Neoplasms: Classification and Clinical Significance

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Recent technological advances have enabled investigators to characterize the molecular genetics and genomics of hepatic neoplasia in remarkable detail. From these studies, an increasing number of molecular markers are being identified that correlate with clinically important tumor phenotypes. This paper discusses current knowledge relevant to the molecular classification of epithelial primary hepatic tumors that arise in adults, including focal nodular hyperplasia (FNH), hepatocellular adenoma (HCA), hepatocellular carcinoma (HCC), cholangiocarcinoma (CC), and combined HCC-CC. Genetic analysis has defined molecular subtypes of HCA that are clinicopathologically distinct and can be distinguished through immunohistochemistry. Gene expression studies have identified molecular signatures of progression from dysplastic nodules (DNs) to early HCC in cirrhosis. Analyses of the mutational spectra, chromosomal aberrations and instability, transcriptomics, and microRNA profiles of HCC have revealed the existence of biologically distinct subtypes of this common malignancy, with prognostic implications. Molecular characterization of biliary and hepatic progenitor cell phenotypes in liver cancer has shed new light on the histogenesis of these tumors and has focused attention on novel therapeutic targets. In coming years, the molecular classification of hepatic neoplasms will be increasingly valuable for guiding patient care, as targeted therapies for liver cancer are developed and brought into clinical practice.

1. Introduction

Over the past decade, tremendous advances have been made in the technologies available for characterizing the molecular genetics, genomics and epigenetics of neoplasia. These advances have greatly accelerated basic research aimed at elucidating the molecular mechanisms underlying tumorigenesis. In addition, they have led to the identification of molecular markers that correlate with important biological characteristics of tumors. Such markers are increasingly valuable in clinical practice as tools for facilitating the diagnosis and categorization of tumors, determining their aggressiveness and, in some cases, predicting their responses to particular forms of therapy. The ideal classification system for any group of neoplasms would be based on our understanding of their ontogeny and would integrate histologic features with molecular data in a clinically meaningful way. In this paper, we will discuss the molecular biology of primary hepatic tumors, with particular emphasis on the roles of molecular characterization in clinical practice. We will focus on primary hepatic neoplasms that arise in adults, including hepatocellular adenoma (HCA), hepatocellular carcinoma (HCC), cholangiocarcinoma (CC), and the rare entity, combined hepatocellular and cholangiocarcinoma (HCC-CC). Focal nodular hyperplasia (FNH), although thought to be a nonneoplastic lesion, is often considered in the differential diagnosis of other hepatocytic tumors and, therefore, has also been included in this discussion. However, consideration of mesenchymal, hematopoietic and childhood liver tumors is beyond the scope of this review. FNH is the most common of the benign hepatocellular tumors, arising in approximately 3% of adults [1, 2]. Although the other major type of benign hepatocellular tumor, HCA occurs much less frequently, with an estimated incidence in Europe and North America of 0.003% [3], its association with hemorrhage, rupture, and risk of malignant transformation necessitates its accurate diagnostic distinction from FNH. This is an area in which molecular
characterization has become very informative and clinically useful. The most common primary hepatic malignancy is HCC, which constitutes 80–85% of all malignant epithelial neoplasms originating in the liver. HCC often arises in a background of cirrhosis and presents a global public health problem far greater than that of HCA. In the United States, the annual incidence of HCC has been rising over the past three decades [4]. The second most common primary hepatic malignancy is CC, constituting 15–20% of the total, and its incidence has also been rising. There has been an explosion of basic research in the field of primary hepatic epithelial cancers in recent years, and we are just beginning to understand their molecular pathogenesis. We anticipate rapid advancements over the next several years in personalized medical approaches to the treatment of liver cancer, as the molecular mechanisms of hepatic tumorigenesis are elucidated and molecular classification of these lesions is integrated into clinical study design [5].

2. Focal Nodular Hyperplasia

Focal nodular hyperplasia (FNH) is a common, benign hepatocellular lesion that is believed to arise in response to localized hyperperfusion of the liver parenchyma, typically in association with a microscopic arterial malformation [6, 7]. FNH frequently presents as an incidental hepatic mass noted in imaging studies, and it can often be diagnosed on the basis of its radiographic appearance alone. However, sometimes the diagnosis requires histologic confirmation. Although the gross morphology and microscopic appearance of FNH in resection specimens are usually characteristic and easily recognized, distinguishing FNH from HCA in needle biopsies can be difficult. The distinction is, however, of great importance because of the differing clinical management of these entities.

Traditionally, FNH has been considered nonneoplastic and, accordingly, it is thought to have no malignant potential. However, molecular analyses of the clonality of FNH have yielded conflicting results, with some studies finding that up to 50% show X chromosome inactivation patterns suggestive of monoclonality [7, 8]. In general, polyclonality argues strongly against neoplasia, but the implications of monoclonality are less clear, since it may be influenced by factors such as variability in X-inactivation patterns, tissue architecture and turnover rate. Thus, the question of whether classic FNH is sometimes neoplastic remains controversial. Atypical forms of FNH have been described, including mixed lesions with both FNH-like and HCA-like areas, as well as lesions with histologic features intermediate between FNH and HCA [9]. The latter tumors were originally designated “telangiectatic FNH” (T-FNH), but subsequent molecular analyses have led to their reclassification as a variant of HCA (see below). Mixed FNH-HCA have not been subjected to molecular analysis and, indeed, have not been described in recent publications. Therefore, their existence as a distinct entity is uncertain. Although FNH and variants of HCA comprise a histologic spectrum (Figure 1), there is no compelling evidence that they are biologically related in the sense of one being the precursor of another.

Given the uncertainty surrounding the issue of neoplasia in FNH, several investigators have searched for genetic alterations that might define these lesions. If a genetic abnormality characteristic of FNH were found, it would greatly support its neoplastic nature, and the finding could be diagnostically useful in clinical practice. However, the results of such studies thus far have been uniformly negative, with no mutations detected in the APC, axin, β-catenin, HNF1α, or p53 genes in classic FNH [8, 10–12].

Both FNH and HCA consist predominantly of well-differentiated hepatocytes. In FNH, there are intervening fibrous bands that radiate from a central scar and contain abundant, proliferating bile ductules. In contrast, HCA have only rare bile ductules, if any, and typically show much less fibrosis. However, the regions of fibrous scar in FNH are variable in abundance and are not always adequately sampled in needle biopsies, sometimes making the histologic distinction from HCA difficult. We have found that the expression patterns of biliary markers, as determined by immunohistochemistry, are frequently different in FNH and HCA, not only within regions containing bile ductules, but also in hepatocellular areas [13]. Antibodies against cytokeratin 19 (CK19) and neuronal cell adhesion molecule (NCAM, also known as CD56), which are markers of hepatic progenitor cells as well as biliary epithelium, clearly stain the proliferating ductules within fibrous tracts in FNH and rare, isolated ductules in HCA. Cytokeratin 7 (CK7), which is known to be a marker of immature hepatocytes

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**Figure 1:** Schematic illustration of the histologic spectrum of benign hepatocellular proliferations. Polyclonal FNH is at one end of the spectrum and nontelangiectatic HCA is at the other; inflammatory HCA (I-HCA), formerly classified as telangiectatic FNH (T-FNH), has overlapping features and is shown in the middle. Gray areas indicate fibrosis; green and red dots represent bile ductules and arterioles, respectively.
as well as progenitor cells and biliary epithelium [14], shows a distinct pattern of expression in HCA [15] that distinguishes the latter from FNH in most cases (Figure 2). In our experience, although occasional needle biopsies remain diagnostically challenging even after the combined use of immunohistochemical stains for CK7 and CK19, this analysis is usually quite helpful in the differential diagnosis of FNH and HCA [13, 16].

Immunohistochemical staining for the cytoplasmic enzyme, glutamine synthetase (GS), has also been proposed as a technique for differentiating between FNH and HCA [2]. GS is an enzyme that combines glutamate with ammonia to produce the amino acid glutamine. In normal liver, GS expression is limited to the centrilobular hepatocytes that directly border central veins, and its most important function is to aid in ammonia detoxification [17]. In recent years, the Wnt signaling pathway has been identified as a critical regulator of zonation in the liver [18]. 

β-catenin activation in perivenular hepatocytes, presumably the result of Wnt signaling from central veins, drives the expression of GS. In FNH, the centrilobular zones of β-catenin activation are expanded [12], and this leads to an irregular, geographic distribution of GS overexpression [2]. In HCA and HCC, several different patterns of GS expression have been observed, all distinct from that seen in FNH. As discussed later, activating mutations of the β-catenin gene occur in a subset of HCA. In adenomas with β-catenin mutation, GS is diffusely expressed, whereas in adenomas with normal β-catenin, GS is absent in large areas of the lesion. Unfortunately, however, in the latter type of HCA, irregular and/or centrilobular expression of GS can be present focally, particularly at the periphery of the tumor [2]. Therefore, although GS expression appears to robustly differentiate FNH from HCA in full resection specimens, it is less reliable for making this distinction in needle biopsies.

3. Hepatocellular Adenoma (HCA)

First described a half-century ago, HCA is a rare, benign liver tumor that most commonly occurs in women and has been associated with oral contraceptive use [19]. It is a neoplasm of demonstrated clonal origin [20, 21] and has a small but nonnegligible risk of malignant transformation [22]. Hemorrhage, pain, and rupture are other, more frequent complications of HCA, and their likelihood is proportional to the size of the tumor [1]. Histologically, HCA is a proliferation of mature-appearing hepatocytes arranged in cords one to two cells thick and lacking portal tracts. Isolated arterioles surrounded by hepatocytes and lacking a fibrous sheath (“naked” or “unpaired” arteries) can be seen scattered throughout the lesion. Arterioles accompanied by portal venules may be observed in a few portal-like structures, but these lack bile ducts. In some HCA, occasional isolated bile ductules may be seen, especially when immunohistochemical stains for CK7 or CK19 are applied.

In 2002, Bluteau et al. published the results of a genome-wide search for tumor suppressor genes in HCA [23]. Microsatellite analysis revealed a loss of heterozygosity (LOH) for markers at chromosome 12q caused by a small deletion of this region in five out of ten HCA. The TCF1 gene, encoding hepatocyte nuclear factor 1 (HNF1), was found to reside within the deletion. Because the function of HNF1 as a liver-specific transcription factor was already well established, this gene was pursued as the most likely candidate tumor suppressor affected by LOH in these adenomas. Further investigation confirmed that in ten out of sixteen HCA analyzed, HNF1 was somatically inactivated, either through a combination of gene deletion and mutation or via bi-allelic mutation. Interestingly, germline mutation of the HNF1 gene had been previously discovered to underlie a rare form of familial noninsulin dependent diabetes, MODY3 (maturity-onset diabetes of the young, type 3) [24]. This form of diabetes is associated with an increased risk of hepatic adenomatosis, due to random somatic inactivation of the second, nongermline-mutant HNF1 allele in the liver [25].

Shortly after genetic alterations of HNF1 in HCA were reported, a group in Taiwan published their discovery of activating mutations of the β-catenin gene in a fraction of these tumors. Earlier work by many independent groups had implicated Wnt pathway activation in liver carcinogenesis, and β-catenin mutations have been found in a significant percentage of HCC. To investigate the possibility that aberrant β-catenin signaling plays a role in very early hepatic...
neoplasia, Chen et al. performed directed LOH analysis and genomic sequencing of several Wnt pathway genes [10]. Three of ten HCA were found to bear small, in-frame deletions in one allele of the β-catenin gene, and to express truncated forms of the protein that are predicted to be constitutively activated.

The French group that had originally identified HNF1 inactivation in HCA next expanded their study to include assessment of β-catenin mutation status as well as genotype-phenotype correlations in these tumors [26]. In a collection of 96 HCA, 44 were found to have HNF1 gene inactivation and to display a characteristic histology, including marked hepatocellular steatosis, a lack of cytologic atypia, and an absence of inflammatory infiltrates in the lesion. Beta-catenin mutation was identified in 12 of the 96 tumors and was frequently associated with histologic features suggestive of malignancy, including nuclear atypia and pseudocinar formation. Importantly, β-catenin mutation and HNF1 inactivation were mutually exclusive in these tumors, indicating the existence of at least two distinct, nonoverlapping molecular subtypes of HCA.

In the 1990s, a class of histologically unusual hepatic tumors was described and termed "telangiectatic FNH" (T-FNH) [9]. These lesions lacked a central scar, but because they displayed focal ductular reaction and a nodular architecture, they were categorized as an atypical variant of FNH. However, the sinusoidal dilatation, naked arterioles, and frequent intralesional hemorrhage that typified T-FNH were recognized as being similar to HCA (Figure 1). These peculiar features of T-FNH invited molecular characterization and comparison with both classic FNH and HCA. Two groups performed clonality assays and found a much higher rate of monoclonality in T-FNH than in classic FNH [11, 20]. The angiopoietin mRNA expression pattern, which had been shown to differ between FNH and HCA, was found in T-FNH to resemble that of HCA, and a global proteomic profile of T-FNH matched that of HCA while distinguishing both T-FNH and HCA from classic FNH [20]. Together, these findings strongly suggest that so-called telangiectatic FNH is actually a variant of HCA.

In the genotype-phenotype study described above, HCA that were found not to harbor genetic alterations in either HNF1 or β-catenin were further subdivided into two groups on the basis of the presence or absence of inflammatory infiltrates. The subgroup showing inflammation, named inflammatory HCA (I-HCA), was found to include all lesions formerly classified as T-FNH. Thus, four subtypes of HCA were proposed: (1) HNF1-inactivated HCA (H-HCA), (2) β-catenin-mutated HCA (β-HCA), (3) I-HCA, and (4) nonmutated, noninflammatory HCA [26]. A subsequent study of 93 HCA (47 of which had been previously analyzed) supported this classification system in general, except that β-catenin mutation was discovered to occur in a fraction of I-HCA as well as in noninflammatory HCA [27].

The hypothesis that I-HCA represents a biologically distinct entity was greatly strengthened with the finding of a marked overexpression of acute-phase reactants by the hepatocytes of these lesions [27]. At both the mRNA and the protein level, serum amyloid A (SAA) and C-reactive protein (CRP) are significantly overexpressed in I-HCA relative to nonneoplastic liver. When all types of hepatocellular adenoma are examined, the sensitivity and specificity of SAA overexpression by immunohistochemistry for identifying I-HCA was found to be 94%. The degree of SAA overexpression did not correlate with the extent of inflammation by inflammatory cells, which suggested that acute-phase protein expression might be an intrinsic feature of the neoplastic hepatocytes, whereas inflammatory infiltration might be secondary. To explore this possibility, Rebuffo et al. undertook a genome-wide mRNA expression study, comparing I-HCA to normal liver, and identified a pronounced activation of acute-phase inflammatory signaling in I-HCA [28]. Further inquiry into the potential causes of this inflammatory gene expression signature led to the discovery of mutations in the IL6ST gene, which encodes gp130, a component of the IL-6 receptor, in 60% of I-HCA [28]. These IL6ST mutations lead to ligand-independent activation of the IL-6 receptor, which promotes STAT3 signaling and induces the acute-phase inflammatory response within hepatocytes. The recruitment of inflammatory cells into I-HCA appears to be secondary to gp130-mediated hepatocellular production of the chemokine, CCL20, which attracts immune cells. In this study, all 43 I-HCA were found to show expression signatures indicative of IL-6 pathway activation, even those tumors in which no IL6ST mutation was identified. In I-HCA lacking IL6ST mutation, no other genetic alterations could be identified in various components of the STAT3 signaling pathway. However, gp130 protein levels were elevated in most of these lesions, suggesting that posttranslational control of gp130 was aberrant, perhaps due to occult mutation of a gene that normally regulates gp130’s protein abundance.

In summary, HCA can be assigned to one of four categories [3]. The first is H-HCA and is defined by HNF1 inactivation. This variety accounts for 35–40% of all HCA and occurs almost exclusively in women. Rarely, these tumors arise in the context of familial diabetes (MODY3) and can be multiple. H-HCA can be identified through immunohistochemical staining for liver fatty acid binding protein (LFABP), which is a transcriptional target of HNF1 and is completely absent in the hepatocytes of these lesions. Histologically, H-HCA usually displays marked steatosis but no inflammation, and it lacks cytologic atypia. The second category, I-HCA, comprises over 50% of all HCA. It is characterized by the presence of inflammatory infiltrates, focal ductular reaction, sinusoidal dilatation, and dystrophic arterioles. I-HCA almost invariably shows dramatic overexpression of acute-phase reactants, including SAA and CRP, which can be demonstrated by immunohistochemistry (Figure 3). Although the HNF1 gene is never inactivated in I-HCA, β-catenin mutations are sometimes found [29]. This type of HCA is associated with obesity, smoking, and alcohol consumption and is the most common variety of HCA in men.

The third category of HCA is somewhat controversial and consists of lesions that harbor activating mutations of β-catenin but are noninflammatory; it has been reported
that approximately 10% of adenomas belong to this \(\beta\)-HCA subtype. Beta-catenin activation is most easily assessed through immunostaining for glutamine synthetase, which is diffusely overexpressed in these lesions. Alternatively, immunohistochemical staining for beta-catenin itself may be used to distinguish between tumors without mutation, in which beta-catenin has a membranous localization, and those with mutation, in which an aberrant, activated form of beta-catenin accumulates within nuclei. In both I-HCA and \(\beta\)-HCA, beta-catenin mutation has been associated with a high risk of malignancy. These tumors are common in men, and they often show features, such as pseudoacini and hepatocellular dysplasia, that are frequent in HCC. In fact, because \(\beta\)-HCA may be histologically indistinguishable from well-differentiated HCC [28], their existence as a biologically distinct entity has been called into question. A group in the United States has recently reported their failure to find any examples of \(\beta\)-HCA in a collection of 41 adenomas subjected to molecular analysis [30]. We feel that in well-differentiated hepatocellular tumors, the presence of beta-catenin mutation in association with morphologic features suspicious for malignancy should warrant a pathologic diagnosis of either outright or suspected hepatocellular carcinoma, rather than HCA. This is an area in need of further research to determine the molecular differences, if any exist, between \(\beta\)-HCA and HCC. Such findings would help to elucidate the pathogenesis of HCC and might serve as the basis for a diagnostic test of malignancy in histologically equivocal cases.

The fourth and final category of HCA are those that are noninflammatory (negative for acute phase markers) and do not harbor mutations in HNF1, beta-catenin, or gp130. This is the smallest group of HCA, accounting for only about 5% of the total. Lesions that cannot be fully evaluated due to extensive necrosis and/or hemorrhage are also classified in this group. Whether these noninflammatory, nonmutant tumors represent a truly distinct subtype of HCA, or whether they are biologically related to one or more of the other three subtypes, is an open question that awaits further investigation.

4. Hepatocellular Carcinoma (HCC)

HCC almost always arises in the context of chronic liver disease, usually in patients with cirrhosis. Cirrhosis of any etiology constitutes the predominant risk factor for HCC. Worldwide, infection with hepatitis B virus (HBV) accounts for the majority of cases, whereas in the United States, chronic hepatitis C is the most common predisposing factor. HCC is often diagnosed late in the course of disease, but in developed countries, surveillance programs that use improved radiologic techniques for monitoring liver lesions in patients with cirrhosis are leading to increased rates of early HCC detection.

4.1. Dysplastic Nodules and Early Hepatocellular Carcinoma in Cirrhosis. It is now well accepted that in cirrhotic livers, the vast majority of HCC arise from benign precursor lesions called dysplastic nodules (DN) [31]. Although fully developed HCCs typically show histologic features that are easily recognizable to pathologists, the morphologic distinction between advanced DN and early HCCs is often more subtle and can be very difficult to identify, especially in needle biopsies [32]. As the molecular genetic events that drive the early steps of hepatic carcinogenesis are more fully elucidated, it is anticipated that molecular markers will be found that can help pathologists in making these clinically important distinctions. Indeed, in recent years, molecular analyses of the dysplasia-to-carcinoma pathway in cirrhosis have begun to yield valuable information for the recognition and classification of early hepatic neoplasia.

Several groups have used gene expression profiling to identify “molecular signatures” that can accurately distinguish between DN and early HCC [33–37]. Perhaps the most promising of these studies is that of Llovet et al., which analyzed the expression patterns of 55 hepatocarcinogenesis-related genes in dysplastic nodules and early HCCs of hepatitis C-infected patients. These authors identified a panel of only three genes (GPC3, LYVE1 and survivin) whose mRNA expression levels, as assessed by quantitative reverse transcription-PCR (qRT-PCR), were shown to correctly predict malignancy in 19 out of 20 early HCCs and nonmalignancy in 16 out of 17 DNs. As a test for HCC in this sample set, the sensitivity of this 3-gene expression panel was 95% and its specificity was 94%. Two of the genes in the panel, GPC3 (which encodes glypican-3) and survivin, are expressed by hepatocytes and are upregulated in HCCs, as compared to DN. The third gene, LYVE1, is expressed by endothelial cells and is downregulated in malignancy. All three genes had been previously implicated in hepatic carcinogenesis, and their differential expression levels in DNs and HCCs is most likely independent of the underlying cause of cirrhosis. The advantage of using qRT-PCR to identify this sort of molecular signature is that the method is highly quantitative, much more so than conventional immunohistochemistry, and, therefore, more objective. However, a disadvantage is that it must be performed on pure lesional tissue,
which can be scarce in needle biopsies. Furthermore, the method requires the sacrifice of a small amount of lesional tissue for RNA extraction and thus necessitates the destruction of some potentially valuable histologic information. Nevertheless, in cases with an adequate amount of tissue, this qRT-PCR-based approach has the potential for considerable practical utility. If its predictive accuracy can be shown to extend to lesions that arise in cirrhosis of all etiologies, and if it can be successfully employed for the analysis of formalin-fixed needle biopsies, it may become a valuable adjunct to histology for differentiating between DN and early HCCs.

The gold standard for diagnosis of DN and early HCCs is still histology; in fact, internationally agreed upon histologic definitions of these lesions and a standardized nomenclature have only recently been formulated [32]. This morphologic classification system was developed and refined on the basis of meticulous histologic analyses of entire lesions within surgical resection specimens. It is recognized that needle biopsies of such lesions will often lack areas that are critical for making a histologic diagnosis of malignancy. In particular, the diagnosis of early HCCs may require identifying the invasion of lesional hepatocytes into portal tracts within the surrounding, benign parenchyma. Because biopsies frequently fail to sample such areas, molecular markers capable of distinguishing between dysplastic and malignant hepatocytes are urgently needed. However, because histology is still the gold standard for diagnosis, the immunohistochemical assessment of molecular markers, as an adjunct to standard H&E histology, is generally preferred over nucleic acid analysis in clinical practice.

Many immunohistochemical markers have been assessed individually, but what has emerged as most useful currently is a panel of three markers: GPC3, GS, and HSP-70. Glypican-3 was first identified as a gene whose mRNA is frequently expressed in HCC but not in benign, adult liver, HCA or CC [38]. Numerous laboratories have since confirmed the overexpression of GPC3 in HCC at the mRNA and protein levels. Its utility as an immunohistochemical marker for HCC was first demonstrated by Yamauchi et al., who found diffusely positive GPC3 staining of malignant hepatocytes in 84% of HCCs and only focal, weak staining in a small set of DN [39]. Similar results were obtained by Llovet et al. in the study cited above.

In an effort to augment the sensitivity of GPC3 immunostaining as a molecular test for HCC, and to retain its specificity in distinguishing DN from malignant lesions, Di Tommaso et al. examined the value of adding other tumor markers to the analysis [40]. Heat-shock protein 70 (HSP70) is an antiapoptotic, “stress response” gene that had been found through mRNA expression profiling to be markedly upregulated in early HCC, as compared with adjacent benign liver tissue [36]. Glutamine synthetase (GS) is a metabolic enzyme (now known to be upregulated by β-catenin signaling [41], as discussed above) that had also been shown to be overexpressed in primary liver cancer (Figure 4) [42]. When these three tumor markers, GPC3, HSP70, and GS, were applied as an immunohistochemical panel to a set of benign and malignant nodules that had been resected from cirrhotic livers, positivity for any two of the three markers was found to indicate malignancy with 72% sensitivity and 100% specificity. In a recent study, these authors used the same panel of immunostains to retrospectively analyze needle biopsies of a similar set of lesions and found that for distinguishing between high grade dysplastic nodules and well- or very well-differentiated HCC, the sensitivity of two-marker positivity as an indicator of malignancy decreased from 72% to 49%, while its specificity remained 100% [43]. This reduction in sensitivity may be attributable to sampling error and the somewhat heterogeneous expression patterns of HSP70 and GPC3 proteins within HCC. It would be
interesting to know whether the mRNA expression patterns of GPC3, LYVE1, and survivin suffer from the same sort of intratumoral heterogeneity, which might complicate their use as molecular markers for HCC in needle biopsies.

Although immunohistochemical staining for the GPC3/HSP70/GS panel is currently recommended as the best ancillary technique to aid in the diagnosis of early HCC in difficult needle biopsy specimens [44], the search continues for biomarkers that can increase the sensitivity of this panel and that are easily and reproducibly stainable in tissue sections. Most candidate markers have been discovered through gene expression studies, and beyond their practical diagnostic utility, investigation into their functions in human liver and in model systems has yielded important insights into the mechanisms of hepatic carcinogenesis. A recent example of gene expression profiling leading to this kind of basic insight is provided by Kaposi-Novak et al. in their study of dysplastic nodules and early HCC in cirrhotic liver explants [37]. In addition to identifying molecular signatures able to discriminate between regenerative, dysplastic, and malignant nodules as well as confirming the upregulation of HSP70, GPC3, and several other putative tumor markers in early HCC, these investigators performed a comparative functional analysis of DN and HCCs gene expression signatures. Their results show that the gene expression profile of early HCCs differs from that of DNs in a pattern strongly suggestive of MYC oncogene activation. Interestingly, no mutations in the MYC gene were detected by sequencing of HCC genomic DNA, and MYC itself was not found to be overexpressed. However, a protein called CSN5 (also known as Jab1) that had been previously shown to posttranscriptionally promote MYC activation in breast epithelium [45] was found to be overexpressed in early HCC in parallel with induction of the MYC-regulated gene expression signature. This suggests a mechanistic model of early hepatocarcinogenesis in which an increase in the expression of CSN5 within DN leads to aberrant MYC activation, and thereby drives the progression of these lesions to HCC. If this model proves true, it may have profound implications for the molecular classification, diagnosis, and treatment of early HCC.

4.2. Hepatocellular Carcinoma Arising in Noncirrhotic Liver. The overall incidence of HCCs in patients without cirrhosis is difficult to estimate and varies with geography and the prevalence of risk factors in the population [46, 47]. Most cases occur in association with chronic liver disease, sometimes in a background of hepatic fibrosis that falls short of full-blown cirrhosis. Consequently, the risk factors for HCC development are the same in the absence of cirrhosis as in its presence; they include chronic HBV or HCV infection, chronic alcohol- or toxin-induced liver injury, and nonalcoholic steatohepatitis [46, 48]. Noncirrhotic patients with HCC are more likely than those with cirrhosis to have multiple risk factors. Although the molecular pathogenesis of hepatic malignancy is likely variable, with some genetic alterations more common in lesions that arise in association with particular risk factors than others, there is no strict correlation between the etiology of underlying chronic liver disease and histologic or molecular subtype of HCCs (see below).

Hepatocellular carcinomas that arise in the absence of chronic liver disease or known risk factors for malignancy are almost always a histologic variant known as fibrolamellar HCC (FL-HCC). The great majority of these tumors occur in patients under the age of 35, and they display a characteristic histology, with nests of large, oncocytic, malignant hepatocytes surrounded by thick bands of layered fibrosis. Although the rarity of these tumors has hampered efforts to elucidate their molecular pathogenesis, FL-HCCs have been found to contain unique molecular alterations that distinguish them from the more common forms of HCCs (reviewed in [49]). The diagnosis of FL-HCCs depends mostly on histology, with the application of immunohistochemical markers when needed [50], and the recognition of clinical characteristics typical of this distinct clinicopathologic entity.

4.3. Hepatocellular Carcinoma Heterogeneity. Hepatocellular carcinoma has long been known to display extraordinary genetic complexity and molecular heterogeneity. However, it is not clear which of the many genomic, genetic, and epigenetic alterations found in HCCs are the most critical in driving its molecular evolution and/or defining its biological behavior. For this reason, there is currently no molecular subclassification of HCCs that is widely accepted and routinely implemented in clinical practice. Nonetheless, a host of molecular genetic markers have been found to correlate with clinical parameters, and in some instances, to have independent prognostic value in particular circumstances.

Although in recent years, surveillance of those at high risk of hepatic malignancy has initiated a trend toward earlier diagnosis of liver cancer, a substantial majority of HCC patients still present with locally advanced or metastatic disease [51]. Even among those whose cancer is diagnosed at a stage early enough to allow them to undergo potentially curative therapy (such as resection, percutaneous ablation, or liver transplantation), the rate of tumor recurrence remains significant. Overall long-term survival of patients with HCCs is poor, in part because most suffer from concomitant, underlying chronic liver disease. However, rates of recurrence and metastasis are not uniform, even within stage-matched patient groups, and there is evidence that from their beginnings, HCC tumors show individual variability in their degree of intrinsic biological aggressiveness [52]. In addition, the number of therapeutic options for HCCs is growing rapidly, due to advances in fields such as interventional radiology and transplantation as well as the emergence of a large armamentarium of molecularly targeted antineoplastic drugs [53, 54]. Thus, for HCC patients at all stages, there is a need for prognostic and predictive biomarkers that can aid in the subclassification of these tumors and help to identify patients who are most likely to respond to each available treatment modality.

4.4. Chromosomal Abnormalities and Genetic Mutations in HCC. HCCs generally show a high level of chromosomal instability, and this is a phenotype that is acquired early
in the process of carcinogenesis [55]. Thus, chromosomal aberrations are common in this type of cancer. However, there is an enormous variety of alterations that can occur, and they have been found throughout the genome. Many of these chromosomal alterations are undoubtedly “passenger” changes, rather than the “drivers” of tumor progression. In the past decade, advances in genomics technologies such as the development of array-based comparative genomic hybridization (array CGH) have allowed investigators to map HCC-associated chromosomal alterations at a much higher resolution than was previously possible. A number of chromosomal regions have now been identified in which gains or losses occur frequently in HCCs (reviewed in [56]), and progress is being made in determining which genes are the targets of these recurrent changes. For example, by comparing array CGH data with global gene expression patterns (determined using DNA microarrays) in 49 HCC samples, Patil et al. were able to correlate the recurrent gain in chromosome 8q with Jab1 overexpression [57]. Interestingly, this is the same gene (also known as CSN5) whose overexpression was recently implicated in the activation of MYC that appears to drive the progression of DN to early HCC [37]. Thus, 8q gain may be a common mechanism promoting early hepatocarcinogenesis.

Although high-resolution mapping of chromosomal alterations in cancer is valuable mostly as a research tool, a group in Japan has demonstrated that HCCs can be subclassified into distinct, clinically meaningful groups based solely on their patterns of chromosomal alterations [58]. In this study, hierarchical cluster analysis was performed on the genomic gain/loss profiles of 87 HCCs, and the tumors were found to partition into two classes, termed A and B. Chromosomal alterations were more numerous in cluster A and typically included gains of 1q, 6p, and 8q as well as 8p losses; this group of tumors was associated with poor patient survival. Cluster A was further subdivided into three subgroups, each characterized by specific high-level amplifications (1q and 6p, 8q, and 17q). Cluster B contained two important subgroups, one without frequent chromosomal alterations and another with amplification of 17q. The authors reasoned that by analogy with known gene amplifications in other cancer types, the recurrent amplifications they discovered in HCC subgroups might indicate the presence of important oncogenes and that these oncogenes might be suitable therapeutic targets. Accordingly, the vascular endothelial growth factor A gene (VEGFA) was found to be contained within the amplified region of chromosome 6p in a subgroup of cluster A tumors, and a gene encoding an effector molecule of the mTOR signaling pathway was found to reside within the amplified region of 17q. The frequent high-level copy number gain of the 6p region encompassing VEGFA has been confirmed in a larger set of HCC tumors and correlated with VEGFA overexpression [59]. Both VEGF and the mTOR pathway are promising therapeutic targets in liver cancer. Therefore, the predictive value of amplifications at 6p and 17q in HCCs as molecular markers of response to VEGF- and mTOR-targeted therapies should be further explored in clinical studies.

The quantitative assessment of genomic alterations as an indicator of tumor aggressiveness has proven useful in predicting the risk of HCC recurrence after liver transplantation. In an effort to identify molecular markers of recurrence risk in HCC patients who had undergone transplantation, Marsh et al. analyzed explanted tumors for loss of heterozygosity (LOH) of microsatellite markers at nine tumor suppressor gene loci [60]. They found that the amount of allelic loss demonstrated within a particular tumor, when combined with other parameters such as tumor size and patient gender, correlated significantly with risk of tumor recurrence. In a subsequent study, the panel of microsatellite markers used in this analysis was refined, and a simple measure of the amount of LOH detected in each tumor was developed, called the fractional allelic imbalance (FAI) [61]. The measurement of FAI and determination of the presence or absence of macrovascular tumor invasion form the basis of the Pittsburgh staging system [62], which is reported to more accurately predict the risk of HCC recurrence after transplantation than staging systems based on radiologic and clinical parameters [63].

HCCs have been extensively analyzed for the detection of somatic mutations affecting known oncogenes and tumor suppressors (reviewed in [64]). The gene encoding β-catenin (CTNNB1), which is mutated in approximately 30% of HCC, is the oncogene most frequently activated in this cancer. HCC bearing β-catenin mutations are more likely than their nonmutant counterparts to show chromosome instability, an absence of HBV infection [65], a well-differentiated histology, and cholestasis [66]. However, there are conflicting data in the literature on the question of whether β-catenin mutation in HCC is associated with favorable or unfavorable prognosis [67, 68]. In general, tumor suppressor genes are more often mutated in HCC than oncogenes. The tumor suppressor most commonly mutated in HCC, overall, is TP53 (encoding the well-known cell cycle regulator, p53), but its frequency of mutation varies with geographical location. TP53 mutation in HCC occurs most commonly in Asia and Africa, where the combination of widespread dietary aflatoxin exposure and endemic hepatitis B fosters a high rate of mutagenesis in the liver [69]. In the West, TP53 mutations are considerably less frequent, affecting about 20% of HCCs. TP53 mutation in these tumors is associated with poor prognosis, chromosomal instability, and HBV infection [64].

4.5. Gene Expression Profiling and Molecular Subtypes of HCCs. A large number of studies have examined global gene expression patterns in HCCs using DNA microarray technology (reviewed in [70, 71]). The advantage of this approach is that it provides information about which signaling pathways and cellular processes are activated or suppressed in these tumors, regardless of the mechanisms (e.g., mutational or epigenetic) underlying the aberrant regulation. Indeed, transcriptomic analysis has led to the identification of reproducible HCC subtypes with differing cellular differentiation and biological behavior that correlate well with prognosis.
and may soon allow better patient stratification for treatment than current clinical HCC staging systems.

In 2004, a group led by Dr. Thorgeirsson at the National Cancer Institute published the results of a genome-wide expression study of 91 HCCs showing that the tumors fell into two subclasses with distinct mRNA expression profiles and dramatically different patient survival [72]. The poor survival group, designated A, displayed gene expression signatures of high cellular proliferation and low apoptotic rate. A subsequent study, in which human HCC expression profiles were compared with those of rodent hepatocytes (fetal hepatoblasts, adult hepatocytes, and genetically engineered murine HCC), led to further subdivision of group A HCCs into hepatoblast-like (HB) and mature hepatocyte-like (HC) subgroups [73]. The hepatoblast gene signature was associated with earlier recurrence and worse survival than the hepatocyte signature, and this association was independent of other pathologic variables. The authors speculated that the HB phenotype may reflect the origin of this subset of tumors from adult hepatic progenitor cells (HPCs), which, like fetal hepatoblasts, are able to differentiate into both cholangiocytes and hepatocytes. Consistent with this hypothesis, several well-known HPC markers such as cytokeratins 7 and 19 (CK7 and CK19) were found to be included in the HB gene signature.

The existence of two broad categories of HCCs, one with and the other without a “high-proliferation” gene expression signature, has been confirmed in multiple, independent studies [71]. Most have found that tumors of the high-proliferation group are more aggressive and less histologically differentiated than the group lacking this expression signature. Boyault et al. presented a remarkably detailed refinement of this molecular classification system, in which three subgroups of each major category of HCC were identified (yielding a total of six HCC subtypes) through expression analysis followed by correlation with various genetic, genomic, and clinical factors [74]. Although not yet clinically applicable, this sort of analysis helps to elucidate the biological basis of HCC heterogeneity and provides a foundation for future prospective studies aimed at correlating treatment responses with molecular subtype classification.

The epithelial cell adhesion molecule, EpCAM, is a known marker of fetal hepatoblasts and adult hepatic progenitor cells as well as biliary epithelium. In 2004, Kim et al. performed microarray studies of cirrhotic liver and found that EpCAM is dramatically overexpressed in premalignant lesions as well as in a subset of HCC [75]. Molecular characterization of the EpCAM-expressing HCC subgroup demonstrated a signature of coexpressed genes that included other HPC markers, such as c-kit and CK19 [76]. The further stratification of tumors into four groups according to EpCAM expression and the patients’ α-fetoprotein (AFP) status (positive or negative for elevated serum AFP) was shown to define four prognostic categories, each with a characteristic gene expression pattern. Importantly, this result was replicated in three independent cohorts of HCC (all HBV-related), in one of which the EpCAM analysis was performed by immunohistochemistry, rather than mRNA hybridization. Thus, the EpCAM-AFP classification system has the potential for easy application in clinical practice. It is not yet clear whether the stratification of AFP-positive cases according to EpCAM expression will be clinically useful as a prognostic indicator, since AFP positivity is already well-known to be associated with aggressiveness in HCC [77]. However, there is evidence that EpCAM expression correlates with biological variables that can be targeted therapeutically, and, therefore, it may prove valuable as a predictive marker of responsiveness to antiangiogenic therapies [78], Wnt/β-catenin pathway inhibitors, and/or stem-cell targeted agents such as anti-EpCAM antibodies [79].

Gene expression profiling has yielded novel insights into the biological heterogeneity of HCCs and has particularly highlighted the importance of hepatic progenitor cell/stem-cell characteristics in defining its aggressiveness. In addition, these studies have focused attention on the roles of “cancer stem-cells” in HCCs [79]. However, much remains to be learned about the origins and behavior of hepatic progenitor cell lineages in benign and premalignant conditions, as well as their relationships to cancer stem-cells, cell-population dynamics within tumors, and the clinicopathologic ramifications of stem-cell marker expression in different HCC molecular subtypes (Figure 5). Although it is not yet clear which gene signatures and molecular markers will have the broadest applicability, recent rapid progress in this field implies that the incorporation of molecular data into HCC classification and staging systems in the near future will lead to continued improvements in the clinical management of HCC.

4.6. MicroRNA Profiling of HCCs. MicroRNAs (miRNAs) are short, noncoding RNA molecules that regulate gene expression by binding to specific messenger RNAs and preventing their translation into protein. Because each type of miRNA is able to downregulate hundreds of genes at a time, miRNAs often control entire transcriptional programs that determine fundamental cellular properties and behavior. Accordingly, miRNA profiling has emerged as an extremely valuable method for phenotyping and subclassifying tumors [80]. Compared to conventional gene expression profiling (in which protein-coding, messenger RNAs are examined), miRNA analysis has several advantages. Due to the stability of miRNAs, formalin-fixed samples (rather than frozen tissue) can be used. Furthermore, the interrogation of hundreds of miRNAs (and often significantly fewer) yields as much information as might be gleaned from examining thousands of messenger RNAs.

Many independent groups have conducted comprehensive analyses of miRNAs in HCC, and a plethora of informative miRNA markers have been identified. Many of these miRNA signatures correlate with important biological parameters, such as metastasis [81–83], differentiation [83–85], HBV or HCV infection [86, 87], tumor recurrence [88], and patient survival [89–91]. In addition to providing new candidates for investigation as possible diagnostic, prognostic, and/or predictive molecular markers in HCCs, these studies are opening new avenues in basic research on the mechanisms of hepatocarcinogenesis. For example,
Ji et al. [92] have discovered that miRNA-181 is overexpressed in EpCAM-positive HCC cells and is a critical, functional determinant of the progenitor phenotype in these cells. MiRNA-181 appears to promote a HPC phenotype by downregulating the expression of key transcription factors that mediate the differentiation and maturation of hepatocytes.

It has been shown recently that the reduced expression (silencing) of miRNA-26 in HCC tumors of male patients is associated both with poor survival and response to interferon-alpha adjuvant therapy [89]. Interestingly, miRNA-26 is differentially expressed according to gender in benign liver, with significantly higher expression in women than in men. Among individuals with HCC, those whose benign liver tissue showed low miRNA-26 expression were found to have tumors in which miRNA-26 was even further downregulated, whereas in patients whose benign liver miRNA-26 expression levels were high, tumor levels of this miRNA were unchanged. These findings suggested that miRNA-26 acts as a tumor suppressor in the liver and that its higher expression in females may be a protective factor against HCC and may contribute to the marked gender bias in risk for this malignancy. Intriguingly, an analysis of gene expression in tumors that had undergone miRNA-26 silencing revealed the activation of interleukin-6 (IL-6) signaling networks. This is interesting in light of recent evidence that estrogen-related inhibition of IL-6 signaling underlies the observed female-specific resistance to hepatocarcinogenesis in mice [93]. An even more exciting finding with regard to miRNA-26 in human HCC was that miRNA-26 silencing within tumors identified the subgroup of patients who benefited from postoperative treatment with interferon-alpha after HCC resection in randomized trials [89]. The idea that the tumors most likely to respond to interferon treatment are those in which miRNA-26 silencing has led to increased proinflammatory and IL-6 signaling is intuitively appealing. Future prospective studies will undoubtedly test the efficacy of interferon-alpha in preventing recurrence of miRNA-26-silenced HCC in broader patient populations, as well as its value in combination with other therapeutic regimens. Thus, miRNA-26 promises to become the first predictive molecular marker in HCC that can be used to match individual patients with the treatments most likely to benefit them.

5. Intrahepatic Cholangiocarcinoma and Combined HCC-CC

Cholangiocarcinoma (CC), defined as carcinoma of biliary-type epithelium, can arise in the liver parenchyma or anywhere along the extrahepatic biliary tract. The extrahepatic type (which includes “Klatskin tumors” of the liver hilum) is much more frequent, accounting for up to 90% of CC cases [94]. The histologic appearance of intrahepatic CC (ICC) is the same as that of extrahepatic forms, typically showing malignant glands embedded in a prominent desmoplastic stroma. Although these morphologic features usually make ICC clearly distinguishable from HCC (a distinction that can
be confirmed through staining for the hepatocytic marker, HepPar-1, and the biliary markers, CK7 and CK19), it can be difficult to differentiate between ICC and metastasis to the liver from a nonhepatic primary tumor. A few new molecular markers of biliary differentiation have recently been described [95], but these have not yet been tested for their ability to distinguish ICC from hepatic metastasis.

CC generally presents at a late stage, when curative resection is no longer possible. Unfortunately, this malignancy is highly aggressive and resistant to standard chemotherapeutic regimens. Molecular characterization of CC is in the early investigative stages (reviewed in [96]), and useful prognostic molecular markers have not yet been reported. The need for more research in this area, and for the development of targeted therapies and predictive molecular markers, is well recognized.

Combined HCC-CC is a rare primary liver tumor characterized by the intimate intermingling of histologically unequivocal HCC and CC components [97]. Most cases show, in addition to typical HCC and CC areas, transitional regions containing immature-appearing cells that express hepatic progenitor markers such as EpCAM. When such transitional regions predominate, the tumor is classified as a subtype of HCC-CC, “with stem-cell features” [98]. Three histologic patterns of intermediate/stem-cell distribution have been described in lesions of this category, including a pattern sometimes termed cholangiolocarcinoma, formerly classified as a variant of CC. Although several immunohistochemical studies of HCC-CC have confirmed the expression of various hepatic progenitor cell markers in these lesions [99, 100], their classification is based on histomorphology, rather than positivity for stem-cell markers per se. The fact that many HPC markers are also expressed by biliary epithelium and the recent findings on progenitor cell marker expression by HCC greatly complicate this field. Thus, although there is evidence that HCC-CC arises from a bipotential hepatic progenitor cell, the histogenesis of this rare neoplasm is still debated (Figure 5).

Only a few studies have examined the molecular characteristics of HCC-CC. Zucman-Rossi’s group in France used microsatellite-based LOH analysis to assess chromosome stability in 9 CC, 15 HCC-CC, and 3 HCC/CC collision tumors [101]. Combined HCC-CC was found to show a high degree of chromosomal instability, similar to CC and unlike HCC. Investigators in Germany recently reported the results of CGH analysis of 49 HCC, 22 hepatic CC, and 7 HCC-CC cases [102]. In this study, combined HCC-CC was found to resemble both HCC and CC, showing several of the specific chromosomal gains and losses typical of HCC, while also displaying a high total number of chromosomal imbalances, similar to CC. Woo et al. recently performed gene expression profiling of 70 HCC, 13 CC, and 7 HCC-CC [103]. Results showed that the gene expression pattern of HCC was markedly different from that of CC, and combined HCC-CC clearly clustered with CC. Further analysis led to the identification of a CC gene signature containing many known biliary and hepatic progenitor cell markers. Interestingly, closer examination of the HCC data revealed that a subset of HCC also expressed the CC gene signature; this subset was designated CC-like HCC (CLHCC). Expression of the CC gene signature by HCCs was associated with poor prognosis. However, its prognostic significance was found to be independent of previously described poor-prognostic gene signatures such as that of the hepatoblast subtype of HCC. Thus, there are many layers of complexity regarding hepatic progenitor cell and biliary phenotypes in primary liver cancers. Overall, stem-cell and biliary characteristics seem to correlate with increased tumor aggressiveness, resistance to chemotherapy, and poor prognosis.

6. Conclusions

In recent years, remarkable progress has been made in elucidating the molecular pathology of hepatic tumors. In the area of benign hepatocellular lesions, advances in our understanding of molecular subtypes have led to a new classification system of HCA, with important genotype-phenotype correlations and easy application to routine clinical practice. In the field of early hepatic neoplasia, microarray studies of gene expression have identified simple molecular signatures that can distinguish dysplastic nodules from early HCCs in a background of cirrhosis. Although such expression signatures can be determined by quantitative RT-PCR, the use of immunohistochemistry, which allows the simultaneous assessment of histology and protein expression, is preferred in needle biopsies. Molecular measures of prognosis in patients with HCCs and CC are emerging; in retrospective studies, a variety of molecular techniques have successfully identified subgroups of HCCs with distinct clinical associations, such as prognosis, risk of recurrence after attempted curative treatment, and response to adjuvant interferon therapy. Although these assays are not yet developed for widespread clinical application, their refinement and use for the stratification of patients undergoing treatment in clinical trials will lead to further advances in therapeutic options for liver cancer. The current system of classifying hepatic tumors based on histologic architecture and patterns of differentiation has many limitations. Molecular studies have supported a model of hepatic tumorigenesis in which neoplasms recapitulate various lineages and stages of hepatic progenitor cell differentiation. These discoveries are likely to have an important impact on liver tumor classification and to lead to a newer classification system, based on histologic and molecular features, with improved prognostic and therapeutic significance.

References


Review Article

Molecular Characteristics of Pancreatic Ductal Adenocarcinoma

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Pancreatic cancer is an almost universally lethal disease and despite extensive research over the last decades, this has not changed significantly. Nevertheless, much progress has been made in understanding the pathogenesis of pancreatic ductal adenocarcinoma (PDAC) suggesting that different therapeutic strategies based on these new insights are forthcoming. Increasing focus exists on designing the so-called targeted treatment strategies in which the genetic characteristics of a tumor guide therapy. In the past, the focus of research was on identifying the most frequently affected genes in PDAC, but with the complete sequencing of the pancreatic cancer genome the focus has shifted to defining the biological function that the altered genes play. In this paper we aimed to put the genetic alterations present in pancreatic cancer in the context of their role in signaling pathways. In addition, this paper provides an update of the recent advances made in the development of the targeted treatment approach in PDAC.

1. Pancreatic Ductal Adenocarcinoma

Annually, approximately 43,140 people are diagnosed (incidence 10–12: 100,000) with pancreatic ductal adenocarcinoma (PDAC) in the United States and the mortality rate of 36,800, almost equals this number [1]. PDAC ranks fourth on the list of cancer-related causes of death and despite extensive clinical and scientific effort, the prognosis of this exceptionally lethal disease has not improved significantly over the past decades. Surgical resection, for which only a minority (<20%) of patients qualify due to advanced stage of disease at time of diagnosis, is currently the only chance for cure, improving five-year survival rates from <4% if left untreated to 25–30% after resection [2–4]. Though of marginal impact, chemo(radiation) therapy administered in (neo)adjuvant setting has been shown to increase short-term survival rates in resectable and advanced stage disease [5–7]. Despite subtle progress over the years in terms of therapeutic strategies, no major new treatment options have come forward from numerous clinical trails. Nevertheless, much progress has been made in understanding the pathogenesis of PDAC during the past decades, suggesting that different therapeutic strategies based on these new insights are on the horizon [8–10].

PDAC, like all cancers, is fundamentally a genetic disease caused by alterations in cancer-associated genes. The identification of such specific mutated genes is critical for understanding the pathogenesis of PDAC. Nevertheless, one cannot achieve a reasonable overview by considering only individual genes in a cancer cell because the neoplastic potential of this cell is the end product of mutations in multiple genes and changes in multiple pathways that interact and reinforce each other. The rapidly expanding knowledge of genetic and molecular alterations and their role in pancreatic carcinogenesis has led to the question whether it is possible to design a patient-specific therapy based on the genetic fingerprint of an individual tumor. Since an increasing focus exists on designing these so-called targeted treatment strategies, this paper is aimed to put genetic alterations pancreatic cells undergo during malignant transformation in the context of their role in signaling pathways. In addition, this paper provides an update of
Figure 1: Progression model of pancreatic ductal adenocarcinoma from normal epithelium to invasively growing tumor. The progression is associated with the stepwise accumulation of specific genetic alterations depicted below the pictures.

the most recent advances made in the development of the targeted treatment approach in PDAC.

2. Precursors of PDAC

The development of invasive carcinoma in the pancreas is a stepwise process. Similar to colon cancer, noninvasive stages have been identified in PDAC preceding invasive carcinoma [11]. In recently published research, the clonal evolution of the earliest genetic alterations in tumor initiating cells towards frankly invasive and metastasized PDAC was followed and these studies indicated that such tumor progression takes at least more than a decade [12, 13]. This creates an important window of opportunity for early detection and much effort is put into attempts to map the genetic changes that take place in the pancreatic ductal cells of precursor lesions before they become invasive.

Since 2004, there have been clear guidelines for classifying these precursor lesions of PDAC and three different types have been identified: pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasia (MCN), and intraductal pancreatic mucinous neoplasia (IPMN) [14]. MCN and IPMN are considered separate and specific entities that fall beyond the scope of this review [15, 16]. By far, the most common and also the generic precursor lesion of PDAC is the PanIN lesion. PanINs are found in the smaller pancreatic ducts and based on the degree of dysplasia reflected in the cytonuclear atypia and architectural change can be classified in four grades: PanIN-1A, PanIN-1B, PanIN-2, and PanIN-3. The least severe abnormalities are seen in PanIN-1 lesions; minimal cytonuclear atypia is present and cell polarity is retained with a basally located nucleus. The difference between PanIN-1A and -1B is that the cells in PanIN-1A lesions are flat, whereas the cells in PanIN-1B lesions are arranged in a micropapillary architecture.

PanIN-2 lesions are characterized by evident cytonuclear atypia and infrequent mitoses. PanIN-3 lesions, also called carcinoma-in-situ, demonstrate all of the hallmarks of cancer including loss of polarity, nuclear atypia, frequent mitoses, and budding of groups of cells in the lumen. Yet, the lesion is confined within the basement membrane and no invasive growth is present [14]. The increasing grades of dysplasia in the various PanIN lesions manifest the morphological steps of tumor progression that precede invasive PDAC. These consecutive steps of tumor progression are genetically accompanied by a cumulative occurrence of specific and generalized molecular genetic alterations. Typically, an interplay between mutations in tumor-suppressor genes, oncogenes, and genome maintenance genes ultimately results in the development of PDAC.

Telomere shortening is considered the initiating event in pancreatic tumorigenesis by inducing genetic instability and is discussed separately below. Another early event in PDAC development is mutation of the oncogene KRAS2, which is found altered in 20% of PanIN-1 lesions and this percentage increases with progression to invasive carcinoma. The tumor suppressor gene most commonly found mutated in PDAC is CDKN2A. This gene is found mutated in, respectively, 30%, 50%, and 70% of PanIN-1, PanIN-2, and PanIN-3 lesions [17]. Two additional important tumor suppressor genes in PDAC are TP53 and SMAD4. In precursor lesions, mutations in these genes are mainly observed in PanIN-3 lesions in transition to invasive growth warranting TP53/SMAD4 defects as a late event in PDAC development [18, 19]. In Figure 1 and Table 1, the most commonly observed specific genetic alterations in preinvasive lesions of PDAC are mentioned, but many additional alterations exist. In order to understand pancreatic carcinogenesis, the whole known spectrum of alterations has to be considered as well as the cellular interactions.
Table 1: Most commonly affected genes in PDAC.

<table>
<thead>
<tr>
<th>Type</th>
<th>Gene</th>
<th>Cellular function</th>
<th>Affected in PDAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor suppressor genes</td>
<td>CDKN2A/p16</td>
<td>G1-S phase cell cycle inhibition</td>
<td>95%</td>
</tr>
<tr>
<td></td>
<td>SMAD4</td>
<td>TGFβ</td>
<td>55%</td>
</tr>
<tr>
<td></td>
<td>TP53</td>
<td>Cell-cycle arrest</td>
<td>75%</td>
</tr>
<tr>
<td>Oncogenes</td>
<td>KRA S2</td>
<td>ERK-MAP kinase signaling</td>
<td>&gt;90%</td>
</tr>
<tr>
<td></td>
<td>CyclinD</td>
<td>Cell cycle progression</td>
<td>65%</td>
</tr>
<tr>
<td></td>
<td>BRAF</td>
<td>ERK-MAP kinase signaling</td>
<td>5%</td>
</tr>
<tr>
<td>Genome maintenance genes</td>
<td>MLH1/MSH2</td>
<td>DNA damage (mismatch) repair</td>
<td>4%</td>
</tr>
<tr>
<td></td>
<td>BRCA2</td>
<td>DNA damage repair</td>
<td>7–10%</td>
</tr>
</tbody>
</table>

3. Molecular Characteristics and Regulatory Pathways in PDAC

In 2008, Jones et al. used global genomic sequencing to identify the genetic alterations in pancreatic cancer cells. Over 21,000 genes were screened in 24 different PDAC samples. On average, 63 relevant genetic alterations were found per sample, emphasizing the extreme complexity of this disease. These genetic alterations mostly affected 12, partially overlapping, signaling pathways that consequently contained abnormalities in the majority of cases [20, 21]. The identification of these pathways intelligently created a comprehensible view of pancreatic carcinogenesis without simplifying too much [21]. All previously known genetic alterations were included and put into the context of the pathways in which they function. Five of the pathways describe specific cellular functions; apoptosis, DNA damage repair, G1/S phase cell cycle progression, cell-cell adhesion and invasion (Figure 2). The other pathways are signaling cascades and can be divided into three groups: embryonic signaling pathways, the MAPkinase signaling pathways, and TGF-β signaling. The molecular characteristics of PDAC are described within the context of these various specific pathways in the subsequent paragraphs. Table 2 gives an overview of the various affected pathways and their most commonly mutated genes in PDAC.

Table 2: The 12 commonly affected signaling pathways in PDAC accompanied by the most commonly affected genes from these pathways.

<table>
<thead>
<tr>
<th>Regulatory pathway</th>
<th>Affected genes</th>
</tr>
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<tbody>
<tr>
<td>Apoptosis</td>
<td>TP53</td>
</tr>
<tr>
<td>DNA damage repair</td>
<td>TP53</td>
</tr>
<tr>
<td>G1/S transition</td>
<td>CDKN2A/p16, CyclinD</td>
</tr>
<tr>
<td>Cell-cell adhesion</td>
<td></td>
</tr>
<tr>
<td>Regulation of invasion</td>
<td></td>
</tr>
<tr>
<td>Integrin signaling</td>
<td></td>
</tr>
<tr>
<td>Homophilic cell adhesion</td>
<td>CDH1</td>
</tr>
<tr>
<td>Embryonic signaling</td>
<td></td>
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<tr>
<td>Notch pathway</td>
<td></td>
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<tr>
<td>Hedgehog pathway</td>
<td></td>
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<tr>
<td>Wnt pathway</td>
<td></td>
</tr>
<tr>
<td>MAPK signaling</td>
<td></td>
</tr>
<tr>
<td>c-Jun N-terminal kinase</td>
<td>KRAS2</td>
</tr>
<tr>
<td>ERK</td>
<td></td>
</tr>
<tr>
<td>TGF-β signaling</td>
<td>SMAD4</td>
</tr>
</tbody>
</table>

create the genetic instability that facilitates tumor development [26]. Telomerase, the gene that maintains telomere length, shows low expression during early pancreatic tumorigenesis before markedly increasing in the invasive tumor. The re-expression of telomerase probably restores genomic stability, enabling tumor progression by preventing further, possibly lethal, chromosomal damage [25, 27].

3.1. Telomere Shortening. Telomere shortening is encountered in virtually all precursor lesions and invasive carcinomas [23, 25]. Telomeres are repeat sequences at the end of linear chromosomes that prevent fusion between the ends of these chromosomes. Pathologically short telomeres can result in ring and dicentric chromosomes that form so-called anaphase bridges during mitosis. Breakage of these anaphase bridges generates highly recombinogenic-free DNA ends, which in turn can result in chromosomal rearrangement. These cycles of chromosome bridging and breakage, called anaphase bridge-breakage-fusion cycles, repeat and thereby

3.2. Apoptosis. Apoptosis, or programmed cell death, plays an essential role in cancer development since resistance to apoptosis is a key factor in the survival of a cancer cell (Figure 3). Apoptosis is induced by executioner caspases upon activation by the apoptosome complex. This complex consists, among others, of cytochrome C, Caspase-9, and Apaf1 and is released from the mitochondria when pro-apoptotic signaling by Bax/Bak overweights antiapoptotic signaling by Bcl2, Bcl-X(L) or Mcl-1. Inhibitors of apoptosis proteins (IAPs) can inhibit apoptosis at the end of the signaling cascade by direct inhibition of the executioner caspases. In PDAC, genes implicated in the apoptosis pathway were found altered in all tumors studied [21]. Also, previous reports document impaired apoptotic signaling
in this disease. For example, a high fraction of apoptotic cells has been correlated with longer overall survival as well as absence of nodal involvement [28]. Moreover, most chemotherapeutics act through apoptosis induction whereby therapy resistance often is the result of defective apoptosis pathways. Antiapoptotic genes BCL-2, BCL-X(L), and MCL-1 are expressed in, respectively, 13%, 54%, and 86% of PDAC samples as shown by immunohistochemistry, and repression of BCL-2 and MCL-1 was shown to enhance apoptosis in PDAC [29, 30]. The observed apoptotic effect was even more pronounced when treatment was combined with gemcitabine [31].

NF-κB is a transcription factor that regulates several different cellular mechanisms, of which most importantly apoptosis. NF-κB stimulates antiapoptotic signaling by targeting BCL-2 and BCL-X(L) [32–34]. The NF-κB signaling pathway is activated by a variety of different mechanisms in PDAC amongst others oncogenic K-ras signaling [35].
3.3. DNA Damage Repair. DNA damage control genes are responsible for safeguarding the integrity of DNA as they code for proteins that repair any damage that occurs in the cell during its lifespan. An important DNA damage repair gene is TP53, a tumor suppressor gene located on chromosome 17p that is frequently disrupted in many different human malignancies. TP53 expression is lost in 50–75% of PDACs [42, 43]. P53 is involved in the cellular response to genotoxic stress where it mediates cell cycle arrest and apoptosis upon DNA damage. Therefore, loss of TP53 signaling results in a decrease in apoptosis and increases the opportunity for genetic alterations to accumulate in the cells.

Germline BRCA2 gene mutations are responsible for ~10% of familial pancreatic cancer but mutations in this gene are also observed in approximately 7–10% of sporadic PDAC. The BRCA2 protein is involved in DNA damage repair, especially interstrand cross-linking repair [44–46]. The BRCA2 gene will be further discussed in the paragraph on hereditary PDAC.

A third group of DNA damage repair genes involved in the development of PDAC is the mismatch repair family (MMR) of genes. The MMR proteins target base substitution mismatches and insertion-deletion mismatches that arise as a result of errors occurring during replication. Alterations in the mismatch repair genes MLH1, MSH2, MSH6, and PMS2 eventually lead to microsatellite instability (MSI) and this genetic instability makes the genome vulnerable for the accumulation of other, more specific genetic alterations. Tumors of the pancreas with MSI are relatively rare compared to other malignancies of the digestive tract and are found in only 5% of pancreatic carcinomas. Pancreatic cancers with MSI have a distinct microscopic morphology that resembles their counterpart in the colon and are similarly called medullary type carcinomas [47–49]. Remarkably, microsatellite unstable tumors have a significantly better prognosis compared to their microsatellite-stable counterparts [47, 48]. PDACs with MSI exhibit a higher antitumor reaction by T-lymphocytes and this could possibly be the reason for a better outcome [49].

3.4. G1/S Phase Cell-Cycle Progression. Cell-cycle progression and regulation is affected in virtually all cancers as is the case for PDAC. Alterations in genes regulating G1/S-phase transition play an important role in facilitating the uncontrolled growth rate of cancer cells. The most commonly affected tumor suppressor gene in PDAC involved in G1/S phase transition is the CDKN2A gene [50, 51]. This gene is located on the short arm of chromosome 9 (9p21) and is known for its involvement in hereditary melanoma when mutated in the germline [52]. The gene is inactivated in >90% of all PDACs, either by homozygous deletion (40%) or an intragenic mutation combined with loss of heterogeneity of the remaining wild type allele (40%) [50, 53]. Promoter hypermethylation is the cause for loss of CDKN2A function in 15% of the cases [50]. P16, the protein product of CDKN2A inhibits phosphorylation of Rb-1, thereby preventing G1/S transition and acting as an inhibitory cell-cycle regulator [54]. Loss of p16 expression therefore leads to uncontrolled G1/S transition and unregulated cell division, which facilitates tumor progression [55].

Other genes involved in cell cycle progression that occasionally show alterations in PDAC are FBXW7, CHD1 and APC2, although much less frequently than CDKN2A [21, 56].

3.5. Cell Adhesion and Invasion. In normal pancreatic tissue, cells are anchored to each other and their surroundings via multiple connections. A decrease in these interactions can allow cells to detach from their surrounding and migrate/metastasize. As such, cell to cell adhesion and interaction play an important role in carcinogenesis. The connection between epithelial cells is mostly mediated by the adherent junctions composed of E-cadherin and catenins. E-cadherin proteins interlock with each other in the extracellular space, while intracellularly the E-cadherin protein is bound to actin filaments through catenins. Reduced expression of E-cadherin and α- and β-catenins was demonstrated in approximately 60%, 40%, and 60% of pancreatic cancer samples, respectively [57, 58]. Reduced expression of E-cadherin is correlated with tumor dedifferentiation and correlates with tumor stage and lymph node involvement [57, 59]. Not only is the interaction between epithelial cells important in the preservation of the integrity of the tissue, the interactions between the neoplastic cells and extracellular matrix also play an important role, especially in PDAC, because stromal tissue surrounds most tumor cells. Integrins comprise a large family of cell surface receptors and they act as a bridge between the extracellular matrix (ECM) and the cytoskeleton [60]. These integrins direct cell migration and play an important role in invasion. In addition to this, integrins also regulate cell proliferation and apoptosis. Integrin-ECM interactions are vital for cell survival but since apoptosis pathways are often affected in PDAC, loss of the interaction does not necessarily lead to apoptosis in cancer cells. Integrin signaling can activate the ERK, JNK MAPK pathway and the PI3K pathway, important pathways in pancreatic tumorigenesis. In approximately two-thirds of the PDAC cases, a defect in integrin signaling can be identified.
3.6. MAPK Signaling Pathways. There are three major mitogen-activated phosphorylated kinases (MAPK): extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38. All MAPK signaling pathways consist of the same basic kinase components. Stimulation of an upstream MAP2K kinase (MAP3K) by growth factors, stress, or other extracellular signals leads to phosphorylation of a MAPK kinase (MAP2K), culminating in the phosphorylation of a terminal MAPK (Figure 4).

The most influential of the three MAPK pathways in PDAC is the ERK pathway. It consists of the Raf protein (MAP3K) that phosphorylates MEK (MAP2K), which in turn phosphorylates ERK (MAPK), the latter influencing transcription of different target genes. This signaling cascade results in the activation of multiple oncogenic cellular functions. The most commonly mutated oncogene in PDAC is the KRAS2 gene, of which the protein product Ras is an upstream activator of ERK signaling. KRAS2 is located on chromosome 12p and the protein has an intrinsic GTPase activity. In PDAC, the gene is virtually always activated by a point mutation in codon 12, the GTP binding domain, leading to a constitutively active Ras protein [65, 66]. Therefore, it can be considered a molecular switch that in this fashion remains in the “on” position firing its oncogenic stimuli. As said before, KRAS2 gene mutations are an early phenomenon in the development of PDAC, and the KRAS2 gene is mutated in ~95% of PDACs. Interestingly, the few tumors that contain wild-type KRAS2 often have a mutation in the BRAF gene, an oncogene located on chromosome 7q. The BRAF gene is
mutated in approximately 5% of the PDACs and Raf, the protein product of \textit{BRAF}, is a downstream target in the Ras signaling pathway. This explains the mutually exclusive nature of \textit{KRAS2} and \textit{BRAF} mutations in PDAC [56]. The high frequency and early nature of \textit{KRAS2} mutations suggest an initiating role in PDAC development as confirmed in several studies on genetically engineered mice [67, 68]. Besides the effects Ras has on the ERK-pathway, Ras also influences multiple other genes among which NF-κB and PI3K/Akt as discussed above.

The second MAPK pathway often affected in PDAC is the JNK pathway. In the whole genome sequence analysis study by Jones et al. mentioned earlier, in all but one of the sequenced samples a genetic alteration in the JNK pathway was identified [21]. In this signaling cascade the MAP3Ks, Ask1, MEK, JNK, and MLK phosphorylate the MAP2Ks, MKK4, and MKK7 which in turn phosphorylate JNK. The JNK pathway becomes activated upon cellular stress but more importantly, the pathway is activated by proinflammatory cytokines such as tumor necrosis factor α (TNF-α) or interleukin 1 (IL1) [69]. MKK4 expression is lost in approximately 4–15% of PDACs [70, 71]. Remarkably, the JNK pathway has both tumor suppressor and oncogenic functions that have to be further investigated. Also it should be noted that the Kras and the JNK pathways intersect; phosphorylation of JNK is partly responsible for induction of angiogenesis through Kras [72]. Recent studies have also connected MKK4 and its downstream targets (JNK and p38) to the TGF-β pathway.

3.7. TGF-β Pathway. The transforming growth factor β (TGF-β) pathway has been linked to PDAC for many years. TGF-β signaling is involved in a wide range of cellular processes [73]: It is one of the most potent cell proliferation inhibitors and has many other cellular responsibilities including differentiation, apoptosis, and angiogenesis [74]. Binding of a TGF-β family ligand to the TGF-βRII receptor leads to phosphorylation of the TGF-βRI receptor and thereby activation of the TGF-β receptor substrates capable of signal transduction, that is, the Smad family proteins. Eight different \textit{SMAD} genes have been described. Once activated, the receptor subsequently phosphorylates a regulatory Smad (Smad1–3, 5, 8), allowing this protein to associate with Smad4. The latter aids the regulatory Smad complex in its transfer to the nucleus where subsequent transcription of the target genes is induced. Inhibitory Smads regulate Smad-signaling through inhibition of the TGF-β receptor phosphorylation. Thus far, Smad7 is the only characterized inhibitory Smad (Figure 5) [73].

TGF-β influences cellular proliferation through inhibition of G1/S-transition. This is accomplished though expression of cyclin kinase inhibitors such as p15, p21, and p27 [75]. Also, TGF-β signaling represses c-Myc expression, an ubiquitous promoter of cell cycle progression. Jones et al. found altered TGF-β pathway expression in all their PDAC samples [21]. The most commonly affected protein in the TGF-β pathway is Smad4. Smad4 is inactivated in ∼55% of PDACs [76, 77]. Patients with preserved Smad4 signaling have a significantly longer survival than patients with Smad4 loss [77–79]. Also, Iacobuzio-Donahue et al. found a significantly higher percentage of Smad4 loss in patients who had died from PDAC with widespread metastatic disease compared to patients who died of locally advanced tumors [79]. Loss of Smad4 expression is not only a prognosticator but it can also serve as a diagnostic biomarker since sensitive and specific antibodies are available that can be used to characterize Smad4 protein expression by immunohistochemistry (Figure 6) [78].

Other proteins in the TGF-β pathway that are occasionally found altered in PDAC are the TGF-βRII (4%) and TGF-βRI (1%) [80]. Apart from binding to the TGF-βR, TGF-β ligands can also activate other signaling pathways including the MAPK pathways ERK and JNK [81–83]. This depicts the fact that although TGF-β signaling has a tumor suppressive function in the normal epithelium, it can promote tumor progression in late disease stages. Further research has to be conducted to determine the true potential of this pathway for the development of targeting agents.

3.8. Embryonic Pathways. Not surprisingly, since embryogenesis shares many characteristics with carcinogenesis, different embryonic pathways are involved in tumor development. There are three embryonic pathways involved in pancreatic organogenesis, but after formation of the pancreas, signaling is largely restricted to a putative progenitor population known as centroacinar cells [84–87]. Several studies have shown upregulation of Notch pathway activity in PDAC and inhibition of this pathway resulted in decreased tumor proliferation and increased apoptosis [87–90]. Somatic point mutations in one of the four Notch-receptor genes do not seem to be the driving force behind altered Notch signaling in pancreatic cancer [90]. Still, 100% of the PDAC samples in a genome-wide sequencing study revealed alterations in the Notch pathway [21]. Notch signaling interacts with many other oncogenic pathways including the Hedgehog pathway, KRAS signaling and the NF-κB pathway.

The second embryonic pathway often affected in PDAC is the Hedgehog (Hh) pathway. This signaling cascade plays an important role in the organogenesis of the gastrointestinal tract. Surprisingly, Hh signaling is absent in the developing pancreas [91, 92] but the pathway is activated in 70% of PDACs [93]. Some of the Hh signaling targets are components of other signaling pathways involved in PDAC such as Wnt proteins, TGF-β, and CyclinD [94–96]. Although it has long been common knowledge that Hh signaling is active in PDAC, its exact role in tumorigenesis is unclear. It seems that neoplastic epithelial cells do not have the ability to react to Hh signaling. Instead, Hh ligands are expressed in the epithelial cells and it has been suggested that these affect the stromal compartment of a tumor through paracrine signaling. In one particular study, the strong desmoplastic reaction characteristic for PDAC was shown to be further enhanced when Hh signaling was activated [96]. In addition, inhibition of the Hh pathway decreased the total
Figure 5: The TGF-β signaling pathway. The TGF-β signaling pathway is activated by binding of TGF-β to a type II receptor, which facilitates the recruitment and phosphorylation of the type I receptor. This pTGF-βRI activates either SMAD2 or SMAD3 by phosphorylation. The phosphorylated SMAD2/3 forms a complex with SMAD4 and transports to the nucleus where it influences SMAD target gene transcription. SMAD7 can inhibit TGF-β signaling through inhibition of TGF-βRI phosphorylation.

Figure 6: SMAD4 immunohistochemistry. Loss of SMAD4 expression is clearly depicted in the PDAC cells. Arrows: single cell with clear histological changes exhibiting SMAD4 loss, surrounded by SMAD4 wild-type cells.

Volume of orthotopically implanted tumors by inhibiting the stromal component in mice [97]. Another study showed that treatment with an Hh pathway inhibitor produced a clear decrease in tumor growth primarily through a decrease in number of stromal cells [98]. Similarly, disruption of Hh signaling in a transgenic mouse model increased response to chemotherapy [99]. This improved response was due to a diminished desmoplastic reaction and better accessibility of the tumor cells for the chemotherapeutic agent. In short, there seems to be an important role for Hh signaling in the stromal component of PDAC. Moreover, since the desmoplastic reaction has been related to resistance to therapy it warrants further investigation of Hh and its role in the development of PDAC. Clinical trials with inhibitors of hedgehog signaling are in progress.

The third embryonic pathway, Wnt, shows increased activity in approximately 30–65% of PDACs, and an increase in Wnt-target expression correlates with poorer differentiation and poor prognosis [100–102]. Active Wnt expression results in the transcription of different target genes including CyclinD, matrix metalloproteinase 7 (MMP7), and c-MYC. CyclinD is overexpressed in 65% of PDACs and stimulates...
G1/S transition. Expression of this protein is associated with poor prognosis [103]. MMP7, a member of the matrix metalloproteinase family, degrades extracellular matrix proteins, and MMP7 expression is implicated in metastases. Overexpression of this protein is found in practically all PDACs [104]. C-Myc is a transcription factor that regulates thousands of genes involving a large spectrum of cellular functions including cell proliferation, differentiation, death, and tissue reorganization. Amplification of CMYC is identified in 20–50% of all PDACs [105]. The Wnt signaling cascade can be activated through interactions with the Hh, NF-κB, TGF-β, and Notch pathways [106–108].

4. Genetic Susceptibility

Approximately 5–10% of patients with pancreatic cancer have a positive family history for the disease. Having a first-degree relative with PDAC doubles the chance of developing pancreatic cancer compared to individuals without such a history, and the risk increases with increasing number of affected relatives suggesting a hereditary component. Some of these PDACs arise in the setting of a known familial cancer syndrome; however, in most instances the genetic basis for the familial aggregation is not known [109].

To date, at least 5 hereditary disorders that significantly increase the chance of pancreatic cancer development have been described. These include familial atypical multiple melanoma and mole (FAMMM) syndrome, Peutz-Jeghers syndrome, hereditary pancreatitis, familial breast cancer, and other syndromes related to alterations in Fanconi anemia genes, and the Lynch syndrome (Table 3).

The FAMMM syndrome is caused by germline mutations in the CDKNA2A gene. As stated before, this gene is often somatically mutated in sporadic pancreatic cancer. Patients suffering from this syndrome have a 20–34-fold risk of developing PDAC [110]. This risk is especially high when the mutation is a specific 19-base-pair deletion in Cdkn2a: the p16-Leiden deletion [111].

Peutz-Jeghers syndrome is caused by mutations in the STK11/LKB1 gene, a serine threonine kinase involved in a large number of cellular functions, from control of cell polarity to metabolism. Patients suffering from this syndrome have a 132-fold increased risk of developing PDAC [112–114].

Patients with hereditary pancreatitis develop recurrent episodes of pancreatitis, starting at a young age. The syndrome is most commonly caused by mutations in the cationic trypsinogen gene PRSS1 [115]. Another gene that is occasionally found altered in patients with hereditary pancreatitis is the serine peptidase inhibitor SPINK1 [116]. Carriers of either of these mutations have a highly increased risk of developing pancreatic cancer with a lifetime risk of 25–40% at age 60.
The two *BRCA* genes are best known for their role in familial breast and ovarian cancers but *BRCA2* also plays a role in pancreatic cancer development. Carriers of germline *BRCA2* gene mutations have a 3–10-fold increased risk of developing PDAC. A specific interest goes to the Ashkenazi Jewish population as approximately 1% of Ashkenazi Jews are carriers of a founder *BRCA2* mutation, 6174delT [117].

Fanconi anemia is a hereditary cancer susceptibility disorder, with occurrence of multiple haematological malignancies. The Brca2 protein interacts with different Fanconi anemia pathway components, and the corresponding encoding genes, especially *FANCC* and *FANCG*, have also been reported to increase the chance of PDAC development when mutated. Recently, *PALB2*, yet another FANC gene, was reported to be responsible for ∼3% of the cases of familial pancreatic cancer [118, 119]. *PALB2* encodes a protein that enables the localization and binding of Brca2 to sites of double-strand DNA breaks.

Lynch syndrome is caused by germline mutations in a number of DNA mismatch repair genes. Patients suffering from the syndrome have a slightly increased chance of developing pancreatic cancer although there is still some debate about the exact role in PDAC development [120].

Identification of germline mutations in the previously discussed genes is of great importance, not only for screening purposes but also because they could potentially hold therapeutic consequences. Furthermore, no genetic basis for cancer susceptibility is identified in most cases of families exhibiting high numbers of PDAC affected individuals. More research on genetic susceptibility for PDAC will have to be conducted to explain the genetic basis for disease development.

### 5. Treatment of PDAC

Adjuvant chemotherapy after resection of the tumor consisting of gemcitabine has been the treatment of choice since 1997 when it was shown to improve both disease-free and overall survival [5, 6]. Several studies examining the effect of adding other therapeutic agents to gemcitabine have been conducted over the past years with disappointing results [121, 122]. Only the addition of erlotinib showed slight improvement of overall survival [123]. A recently published report confirmed the earlier observed limited beneficial effect of adding erlotinib; however, the authors concluded that this was no justification for a phase III trial [124]. Reports comparing single-agent gemcitabine to adjuvant chemoradiation therapy have been inconclusive. Chemoradiation therapy has been implicated in the USA since the Gastro-Intestinal Tumor Study Group trial was published which showed longer overall survival in patients treated with adjuvant chemoradiation [125, 126]. In Europe, however, a similar study failed to find a significant survival advantage for the group receiving additional radiotherapy thus chemoradiation therapy did not become the standard treatment [127]. Although the most recent reports on this subject suggest a significant advantage for the addition of radiotherapy, there is still controversy about this subject and more research needs to be done before radiation therapy can be included as standard first-line of treatment for PDAC in Europe [125]. It has been suggested that neoadjuvant treatment with chemotherapy, radiation therapy, or chemoradiation therapy, could downstage borderline resectable tumors. Several recent studies have shown promising results for treating borderline resectable tumors with chemoradiation, enabling resection and approaching similar survival rates as truly resectable tumors [128–131]. This is still under investigation and future studies will have to be conducted to justify the use of neoadjuvant treatment.

Approximately 80% of patients present with locally or systemically advanced disease-making resection redundant. For these patients, only palliative treatment options remain. Single-agent gemcitabine is currently recommended as standard first-line chemotherapy for patients with advanced disease [5].

Since the arrival of whole genome sequencing, it has become possible to identify all the genomic alterations that lead to the development of pancreatic cancer. The next logical step is to translate this knowledge into better treatment options. Until recently, no targeted agents were found to improve outcome in the clinical setting although many studies have shown promise in the *in vitro* setting. In the past year, a group used mutation analysis to guide their treatment strategy for the first time [132]. Earlier studies had shown that PDAC cell lines harboring mutations in the above-mentioned *BRCA2* gene, but also other genes related to the Fanconi Anemia syndrome (*FANCC, FANCG*) responded better to treatment with interstrand cross linking (ICL) agents than *FANC/BRCA* wild-type tumors [133]. The *FANC/BRCA* pathway is involved in the repair of double stranded DNA-breaks. As ICL-forming agents induce this type of DNA damage, susceptibility of *FANC/BRCA* mutated tumors to the ICL-forming agents seemed reasonable. Showalter et al. performed mutation analysis for *BRCA2* and one patient harboring a mutation in this gene was treated
with cisplatin, an ICL agent, in addition to gemcitabine showing favorable results (the patient is still alive after 32 months). In theory, PDACs carrying PALB2 mutations should be sensitive to the same targeted therapeutic as PALB2 is a binding partner of BRCA2. Trials justifying use of ICL agents in PALB2 mutated PDAC still have to be conducted.

We have recently seen a Peutz-Jeghers syndrome patient with pancreatic cancer whose tumor showed complete loss of LKB1, an inhibitor of mTOR. This patient responded to treatment with everolimus, one of the known mTOR inhibitors used in clinical setting. Specifically, the tumor diminished in size by more than 50% within 6 months but became resistant thereafter [134].

Inhibition of Kras signaling with farnesyl transferase inhibitors used in the past did not have a beneficial effect (reviewed by [135]). In 2010, a new therapeutic agent was identified targeting the Kras pathway. Protein Kinase C iota (PKC iota) was shown to drive transformed growth in pancreatic cancer cell lines via inhibition of oncogenic Kras activity, and inhibition of PKC iota resulted in a significant reduction of metastases and invasion in preclinical models [136]. Further research has to be done to map the effectiveness of inhibiting PKC iota in vivo.

MTAP, a gene located near CDKN2A, is codeleted with the CDKN2A gene in 30% of the pancreatic cancers. MTAP might be a possible therapeutic target as approaches to selectively target cells with MTAP defects have already been developed [137, 138]. However, these have not been tested in a clinical setting yet.

It seems logical that over the next few years multiple small steps, hopefully adding up to significant progress, will be taken on the road to targeted treatment of PDAC.

6. Conclusion

The aim of this review was to emphasize the complexity of tumorigenesis in pancreatic ductal adenocarcinoma and to provide an introductory overview of the pathways affected in PDAC. As the knowledge on tumorigenesis of PDAC expands rapidly, so do the possibilities to design more effective treatment. The arrival of genome sequencing has offered the opportunity to establish an overview of the genetic alterations that lead to tumor development and this could subsequently play an important role in our search for new therapeutic targets. The complexity of the genetics accompanying PDAC indicates that it is impossible to design a treatment that fits all. From this can be deducted that personalized treatment based on tumor genotyping will probably be most effective and feasible. The use of ICL agents for tumors harboring BRCA2 mutations is the first step in that direction.

Based on the data presented in this review, it seems advisable to shift the focus of research from most commonly affected genes to the most commonly affected pathways as some important yet rare alterations could be missed. The interactions all these pathways undergo are extensive and complex as mentioned earlier. When considering personalizing treatment, designing workable and quick tumor characterizing assays and targeting pathways rather than individual genes seems to hold the future of cancer therapy in PDAC.

Abbreviations

PDAC: Pancreatic ductal adenocarcinoma
PanIN: Pancreatic intraepithelial neoplasia
MCN: Mucinous cystic neoplasia
IPMN: Intraductal pancreatic mucinous neoplasia
PI3K: Phosphatidylinositol 3-kinase
mTOR: Mammalian target of rapamycin
MMR: Mismatch repair
MSI: Microsatellite instability
ECM: Extracellular matrix
ADAM: A-disintegrin and metalloproteinase
ERK: Extracellular signal-regulated kinase
JNK: C-Jun N-terminal kinase
MAPK: Mitogen activated phosphorylated kinases
Hh: Hedgehog
TGF-β: Transforming growth factor β
TNF-α: Tumor necrosis factor α
IL1: Interleukin 1
MMP7: Matrix metalloproteinase 7
FAMMM: Familial atypical multiple melanoma and mole
ICL: Interstrand crosslink
PKC iota: Protein kinase c iota.

Conflict of Interests

Dr. Hruban has received royalties from Myriad genetics for the PALB2 invention.

References


A. J. Aguirre, N. Bardeesy, M. Sinha et al., “Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic...


Review Article

Impact of KRAS Mutations on Management of Colorectal Carcinoma

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The epidermal growth factor receptor (EGFR) pathway is a therapeutic target in the management of colorectal cancer (CRC). EGFR antagonists are active in this disease; however, only a subset of patients respond to such therapy. A Kirsten ras sarcoma viral oncogene (KRAS) wild-type (WT) status of the tumor is necessary, but possibly not sufficient, for a response to anti-EGFR monoclonal antibody therapy. Mechanisms of primary resistance to such therapy in patients harboring KRAS WT tumors are discussed. Strategies to overcome resistance to anti-EGFR monoclonal antibody therapy, including novel agents and combinations of novel therapies, are explored. Also, the use of anti-EGFR monoclonal antibodies in the adjuvant and neoadjuvant setting is reviewed.

1. Introduction

Tumor growth and progression depends in part on the activity of cell surface membrane receptors which control signal transduction pathways. These growth factor receptors can have aberrations in their expression and regulation and activation of growth factor pathways is common in many malignancies [1]. The EGFR, a transmembrane glycoprotein also called ERBB-1 or HER1, is a member of a family of receptor tyrosine kinases (TKs). The EGFR is involved in signaling pathways controlling cell growth, differentiation, and proliferation and is expressed in many different types of normal tissues as well as several tumor types, including CRC [2, 3]. Figure 1 illustrates the main EGFR signaling pathways described [4]. When a ligand binds to the EGFR, the receptor forms a dimer resulting in a signaling cascade within the cell via tyrosine kinase activity [5]. This signaling cascade occurs by the activation of receptor autophosphorylation which triggers a number of intracellular pathways regulating cell proliferation, prevention of apoptosis, and promotion of invasion, metastasis, and neovascularization [6]. The proto-oncogene c-erb-B encodes the EGFR, and activation of the proto-oncogene results in EGFR expression in many tumors [7, 8]. There was therefore interest in investigating this pathway as a potential anticancer therapy target.

Pharmacologically, there are two classes of EGFR antagonists currently in clinical use: anti-EGFR monoclonal antibodies directed against the extracellular domain of the receptor and oral small-molecule EGFR TK inhibitors which block the receptor TK activity competitively [10]. The anti-EGFR monoclonal antibodies, cetuximab and panitumumab, act by binding to the extracellular region of the EGFR and therefore block the ligand-binding region which prevents ligand-induced TK activation [11]. These monoclonal antibodies solely recognize the EGFR, making them very selective for their target [5]. The small-molecule EGFR TK inhibitors, erlotinib and gefitinib, inhibit the catalytic activity of the TK by competing with adenosine triphosphate (ATP) to bind to the intracellular domain [10]. These small-molecule inhibitors are not exclusive to the EGFR pathway and can block different receptor tyrosine kinases, such as the vascular endothelial growth factor (VEGF) receptor and other members of the EGFR family.
Anti-EGFR monoclonal antibodies have been evaluated in both untreated metastatic CRC and chemotherapy refractory disease. Figure 2 summarizes the current treatment paradigm for metastatic colorectal cancer including the appropriate incorporation of anti-EGFR monoclonal antibody therapy which improves survival for appropriately selected patients [9]. Table 1 summarizes selected clinical trials of anti-EGFR monoclonal antibodies in metastatic CRC. Response rates with single-agent anti-EGFR monoclonal antibodies range from 9–12%, with much higher response rates observed when cetuximab is used in combination with chemotherapy [12–22]. When administered to unselected metastatic CRC patients, only a minority responded to EGFR inhibitor therapy. Therefore, a method to identify and predict sensitivity to these drugs was needed.

2. Prediction of Response to Anti-EGFR Monoclonal Antibodies

The RAS family of proto-oncogenes include HRAS, KRAS, and NRAS [23]. KRAS (Kirsten ras sarcoma viral oncogene) is a guanosine triphosphate-(GTP-) binding protein downstream of the EGFR and is a central component of the mitogen-activated protein kinase (MAPK) pathway, a component of the EGFR signaling cascade [23]. Roughly 40% of colorectal cancers are characterized by a mutation in the KRAS gene [24]. About 90% of these mutations occur in codons 12 and 13 in exon 2 of the KRAS gene, with the remaining mutations occurring in codons 61 and 146 (roughly 5% each) [25, 26]. Such KRAS mutations lead to EGFR-independent constitutive activation of the signaling pathway and predict for a lack of response and benefit from anti-EGFR monoclonal antibodies cetuximab and panitumumab [27–35]. De Roock et al. showed that codon 61 mutations predicted for lack of response to cetuximab similar to codon 12 and 13 mutations; however, codon 146 mutations did not affect cetuximab efficacy [26]. Failure to test for codon 61 mutations may miss a significant mutation which would confer resistance to anti-EGFR monoclonal antibody therapy. There is a very high concordance of KRAS mutational status between the primary tumor and metastasis, ranging from 92–100% [36–38]. However, KRAS mutation status heterogeneity between primary tumors, lymph nodes and distant metastases in 5–10% of patients has been reported, with mixed responses to anti-EGFR monoclonal antibody therapy in those with metastatic CRC [37, 39, 40]. Because of this, some clinicians have called for a reassessment of KRAS mutation status on metastatic foci in situations where only the primary tumor was assessed for KRAS status [41].

Table 2 summarizes clinical trials of anti-EGFR monoclonal antibodies which included analysis of treatment effect and KRAS mutational status. Amado et al. assessed the predictive role of KRAS mutational status in a randomized phase III trial comparing panitumumab monotherapy with best supportive care (BSC) in patients with chemotherapy refractory metastatic CRC [24]. This trial showed that the clinical benefit associated with panitumumab was restricted to the KRAS WT population. KRAS mutations predicted for lack of clinical benefit to panitumumab [24]. Similarly, Karapetis et al. showed that treatment with cetuximab significantly improved OS and PFS in patients with KRAS WT tumors; however, in this chemotherapy-resistant patient population, those with mutated KRAS tumors did not benefit [38]. Use of cetuximab as first-line treatment for metastatic disease was investigated by Van Cutsem et al.; patients were randomly assigned to receive FOLFIRI with or without cetuximab [36]. A statistically significant benefit in PFS for patients with KRAS WT tumors receiving cetuximab and chemotherapy was confirmed in a final presentation of this trial [42]. Bokemeyer et al. investigated the use of cetuximab in combination with FOLFOX chemotherapy as initial treatment for metastatic disease [34]. A retrospective analysis of this data revealed that cetuximab and chemotherapy had a statistically significant increased response rate and lower risk of disease progression compared with chemotherapy alone in patients with KRAS WT tumors [43]. Prospectively, panitumumab has been investigated with either FOLFOX or FOLFIRI chemotherapy in the first-line metastatic setting [44, 45]. The addition of panitumumab to FOLFOX chemotherapy was associated with a statistically significant improvement in PFS [44]. Taken together, the data in Table 2 supports that in metastatic CRC, KRAS WT and mutation status predict for potential sensitivity to, and definite resistance to, respectively, both anti-EGFR monoclonal antibodies, regardless of prior treatment and irrespective of use as monotherapy or in combination. Notably, while KRAS status is an established predictor of response to anti-EGFR monoclonal antibody therapy, it has been disproven as a prognostic marker. In contrast to KRAS mutational status, evaluation of EGFR expression of CRC cells has failed to demonstrate predictive value for anti-EGFR monoclonal antibody therapy. Cunningham et al. reported that the intensity of EGFR staining by immunohistochemical analysis did not correlate with response rate to cetuximab [13].

Similar data has also been reported with panitumumab [46]. KRAS mutated CRC absent of anti-EGFR monoclonal
antibody therapy is not inferior to patients with KRAS WT disease. The evaluation of KRAS mutational status is a mandatory aspect of management of patients at the time of diagnosis of metastatic CRC.

3. Mechanisms of Resistance

While KRAS mutations are a major mechanism of primary resistance to antiEGFR monoclonal antibody therapies, resistance mechanisms in KRAS WT patients are also being defined. Forty-sixty percent of patients with KRAS WT tumors fail to respond to treatment with antiEGFR monoclonal antibodies [51]. Therefore, other possible molecular determinants of response are being identified in those patients with EGFR monoclonal antibody-resistant WT KRAS disease.

The importance and frequency of NRAS (a ras oncogene variant) mutations in CRC remains under-investigated [52, 53]. Lambrechts et al. found that NRAS, KRAS, and BRAF mutations were all mutually exclusive events, with combined WT status of these genes associated with higher response rates and longer progression-free survival times [54]. Lambrechts et al. also reported that an NRAS mutation was associated with a lack of response to cetuximab. Irahara et al. investigated the relationship between NRAS mutations and clinical outcome in a collection of 225 colorectal cancers from two prospective cohort studies [55]. NRAS mutations were detected in 2.2% of the CRCs. There was no apparent association between the NRAS mutations and any clinical or pathologic features, including patient survival. However, the low frequency of NRAS mutations may obscure a significant relation. De Roock et al. conducted a retrospective analysis of over 700 tumor samples from patients treated with cetuximab plus chemotherapy and found a NRAS mutation frequency of 2.6%. Additionally, in KRAS wild types, carriers of NRAS mutations had a significantly lower response rate to cetuximab than NRAS wild types (7.7% versus 38.1%, \( P = .013 \)) [26]. There was, however, no significant difference in median PFS (14 versus 26 weeks, \( P = .055 \)) and median OS (38 versus 50 weeks, \( P = .051 \)) between NRAS wild types and mutants [26].

B-type Raf kinase (BRAF) is a component of the RAS-RAF-MEK signaling cascade of the EGFR (see Figure 1) [56]. A specific mutation in the BRAF gene (V600E) is present in approximately 5–8% of CRCs and is thought to be limited to those tumors without mutations in exon 2 of KRAS [42, 57]. BRAF, which is located directly downstream from RAS, can have activating mutations leading to stimulation of the MEK pathway [56, 58]. BRAF mutations appear to confer a poor prognosis, and it appears that BRAF mutations also predict for a lack of response to antiEGFR monoclonal antibodies [42, 57, 59, 60]. Loupakis et al. analyzed 87 patients with KRAS WT tumors for the BRAF V600E mutation who were receiving irinotecan and cetuximab for refractory metastatic colorectal cancer. The BRM V600E mutation was associated with a lack of response to cetuximab and irinotecan, and the response rate was significantly lower in patients with the BRAF V600E mutation compared to those without the mutation (10% versus 38%, \( P = .046 \)) [26].
<table>
<thead>
<tr>
<th>Study</th>
<th>Setting</th>
<th>Treatment</th>
<th>No. of patients</th>
<th>ORR (%)</th>
<th>mTTP (mos)</th>
<th>mPFS (mos)</th>
<th>mOS (mos)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single Arm phase II [12]</td>
<td>Irinotecan-refractory</td>
<td>Cetuximab monotherapy</td>
<td>57</td>
<td>9</td>
<td>1.4</td>
<td>N.R.</td>
<td>6.4</td>
</tr>
<tr>
<td>Randomized phase II [13]</td>
<td>Refractory disease to 5-FU and Irinotecan</td>
<td>Cetuximab monotherapy vs. Cetuximab + Irinotecan</td>
<td>111 vs. 218</td>
<td>10.8* vs. 22.9</td>
<td>1.5* vs. 4.1</td>
<td>N.R.</td>
<td>6.9 vs. 8.6</td>
</tr>
<tr>
<td>Single Arm phase II [14]</td>
<td>Refractory disease to 5-FU, Irinotecan, and Oxaliplatin</td>
<td>Cetuximab monotherapy</td>
<td>346</td>
<td>12.4</td>
<td>1.4</td>
<td>N.R.</td>
<td>6.6</td>
</tr>
<tr>
<td>Randomized phase III [16]</td>
<td>Refractory disease to 5-FU, Irinotecan and Oxaliplatin</td>
<td>Cetuximab monotherapy vs. BSC</td>
<td>287 vs. 285</td>
<td>8* vs. 0</td>
<td>N.R.</td>
<td>1.9* vs. 1.8</td>
<td>6.1* vs. 4.6</td>
</tr>
<tr>
<td>Randomized phase III [18]</td>
<td>Refractory to Oxaliplatin</td>
<td>Cetuximab + Irinotecan vs. Irinotecan</td>
<td>648 vs. 650</td>
<td>16.4* vs. 4.2</td>
<td>N.R.</td>
<td>4.0* vs. 2.6</td>
<td>10.7 vs. 10.0</td>
</tr>
<tr>
<td>Randomized phase III [19]</td>
<td>First-line treatment</td>
<td>Cetuximab + FOLFIRI vs. FOLFIRI</td>
<td>602 vs. 600</td>
<td>46.9* vs. 38.7</td>
<td>N.R.</td>
<td>8.9* vs. 8.0</td>
<td>N.R.</td>
</tr>
<tr>
<td>Randomized phase III [20]</td>
<td>Refractory disease to 5-FU, Irinotecan and Oxaliplatin</td>
<td>Panitumumab monotherapy versus BSC</td>
<td>231 vs. 232</td>
<td>10.0* vs. 0</td>
<td>N.R.</td>
<td>8 weeks* vs. 7.3 weeks</td>
<td>6.5 vs. 6.5</td>
</tr>
<tr>
<td>Randomized phase II [21]</td>
<td>Refractory to Irinotecan</td>
<td>Irinotecan + Cetuximab+ Bevacizumab vs. Cetuximab + Bevacizumab</td>
<td>43 vs. 40</td>
<td>37 vs. 20</td>
<td>7.3 vs. 4.9</td>
<td>N.R.</td>
<td>14.5 vs. 11.4</td>
</tr>
<tr>
<td>Single Arm phase II [22]</td>
<td>Refractory to Irinotecan + Bevacizumab</td>
<td>Cetuximab + Bevacizumab + Irinotecan</td>
<td>33</td>
<td>9</td>
<td>3.9</td>
<td>N.R.</td>
<td>10.6</td>
</tr>
</tbody>
</table>

*Statistically significant improvement.

ORR: overall response rate; mTTP: median time to progression; mPFS: median progression-free survival; mOS: median overall survival; N.R.: not reported; 5-FU: 5-fluorouracil; BSC: best supportive care; FA: folinic acid; NS: not significant.

CRC. This mutation was found in 15% of the patients and was associated with a lack of response to therapy (0% versus 32%, \( P = .016 \)) and a shorter overall survival (4.1 months versus 13.9 months, \( P = .037 \)) [61]. An additional retrospective analysis of 113 patients treated with anti-EGFR monoclonal antibodies found the V600E BRAF mutation in 14% of the KRAS WT patients and was associated with no response to therapy and a statistically significant shorter progression-free survival and overall survival compared with BRAF WT patients [59]. In De Roock’s retrospective analysis of tumor samples from patients treated with cetuximab plus chemotherapy, a BRAF mutation was discovered in 4.7% of tumors [26]. In KRAS wild types, carriers of BRAF mutations had a significantly lower response rate to cetuximab than in BRAF wild types (8.3% versus 38.0%, \( P = .0012 \)), a significantly shorter PFS (8 versus 26 weeks, \( P < .0001 \)), and a significantly shorter OS (26 versus 54 weeks, \( P < .0001 \)) [26]. KRAS and BRAF mutation status do not, however, appear to affect the clinical benefit of oxaliplatin or irinotecan on PFS or OS [62]. Several compounds (PLX4032, PLX4720, and GDC-0879) which selectively inhibit the kinase enzyme BRAF containing the V600E mutation are in clinical development [63]. In BRAF mutant cancer cell lines, these selective BRAF inhibitors potently block RAF-MEK-ERK signaling. However, in those tumors that are BRAF WT, but possess a KRAS mutation, these BRAF inhibitors activate this same pathway and therefore should be avoided in those cancers with RAS mutations [64–66].
<table>
<thead>
<tr>
<th>Study</th>
<th>Setting</th>
<th>Treatment</th>
<th>KRAS genotype</th>
<th>No. of patients</th>
<th>ORR (%)</th>
<th>mPFS (mos)</th>
<th>mOS (mos)</th>
</tr>
</thead>
<tbody>
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<tr>
<td>Lièvre et al. [34]</td>
<td>Second-line treatment</td>
<td>Cetuximab</td>
<td>WT</td>
<td>65</td>
<td>40*</td>
<td>31.4 wk*</td>
<td>14.3*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mut</td>
<td>24</td>
<td>0</td>
<td>10.1</td>
<td>10.1</td>
</tr>
<tr>
<td>De Roock et al. [29]</td>
<td>Irinotecan refractory</td>
<td>Cetuximab or cetuximab + irinotecan</td>
<td>WT</td>
<td>57</td>
<td>41*</td>
<td>34 wk†</td>
<td>12 (cetux)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mut</td>
<td>46</td>
<td>0</td>
<td>12</td>
<td>12</td>
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<tr>
<td>Khambata-Ford et al.</td>
<td>Second or third-line treatment</td>
<td>Cetuximab</td>
<td>WT</td>
<td>50</td>
<td>10</td>
<td>N.R.</td>
<td>N.R.</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Mut</td>
<td>30</td>
<td>0</td>
<td>N.R.</td>
<td>N.R.</td>
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<tr>
<td>Di Fiore et al. [47]</td>
<td>Refractory disease</td>
<td>Cetuximab + chemotherapy</td>
<td>WT</td>
<td>43</td>
<td>20.3</td>
<td>44.7 wk†</td>
<td>27 wk (cetux)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mut</td>
<td>16</td>
<td>0</td>
<td>27 wk (cetux)</td>
<td>25.3–27.3</td>
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<tr>
<td>Benvenuti et al. [48]</td>
<td>Various lines of treatment</td>
<td>Cetuximab or panitumumab or cetuximab + chemotherapy</td>
<td>WT</td>
<td>32</td>
<td>31</td>
<td>N.R.</td>
<td>N.R.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mut</td>
<td>16</td>
<td>6</td>
<td></td>
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<td>Randomized studies</td>
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<td></td>
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<tr>
<td>Amado et al. [24]</td>
<td>Refractory disease</td>
<td>Panitumumab + BSC vs. BSC</td>
<td>WT</td>
<td>124</td>
<td>17</td>
<td>12.3 wk*</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mut vs.</td>
<td>84</td>
<td>0</td>
<td>7.4 wk</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>WT</td>
<td>119</td>
<td>0</td>
<td>7.3 wk</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mut</td>
<td>100</td>
<td>0</td>
<td>7.3 wk</td>
<td>4.4</td>
</tr>
<tr>
<td>Van Cutsem et al. [33]</td>
<td>First-line treatment</td>
<td>FOLFIRI + cetuximab vs. FOLFIRI</td>
<td>WT</td>
<td>172</td>
<td>59.3</td>
<td>9.9*</td>
<td>24.9</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Mut vs.</td>
<td>105</td>
<td>36.2</td>
<td>7.6</td>
<td>17.5</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>WT</td>
<td>176</td>
<td>43.2</td>
<td>8.7</td>
<td>21.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mut</td>
<td>87</td>
<td>40.2</td>
<td>8.1</td>
<td>17.7</td>
</tr>
<tr>
<td>Van Cutsem et al. [42]</td>
<td>First-line treatment</td>
<td>FOLFIRI + cetuximab vs. FOLFIRI</td>
<td>WT</td>
<td>316</td>
<td>57.3*</td>
<td>9.9*</td>
<td>23.5*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mut vs.</td>
<td>214</td>
<td>31.3</td>
<td>7.4</td>
<td>16.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>WT</td>
<td>350</td>
<td>39.7</td>
<td>8.4</td>
<td>20.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mut</td>
<td>183</td>
<td>36.1</td>
<td>7.7</td>
<td>16.7</td>
</tr>
<tr>
<td>Bokemeyer et al. [31]</td>
<td>First-line treatment</td>
<td>FOLFOX + cetuximab vs. FOLFOX</td>
<td>WT</td>
<td>61</td>
<td>61*</td>
<td>7.7*</td>
<td>N.R.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mut vs.</td>
<td>52</td>
<td>33</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>WT</td>
<td>73</td>
<td>37</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mut</td>
<td>47</td>
<td>49</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td>Bokemeyer et al. [43]</td>
<td>First-line treatment</td>
<td>FOLFOX + cetuximab vs. FOLFOX</td>
<td>WT</td>
<td>82</td>
<td>57*</td>
<td>8.3*</td>
<td>22.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mut vs.</td>
<td>77</td>
<td>34</td>
<td>5.5</td>
<td>18.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>WT</td>
<td>97</td>
<td>34</td>
<td>7.2</td>
<td>13.4</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>Mut</td>
<td>59</td>
<td>53</td>
<td>8.6</td>
<td>17.5</td>
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<tr>
<td>Karapetis et al. [35]</td>
<td>Refractory disease</td>
<td>Cetuximab + BSC vs. BSC</td>
<td>WT</td>
<td>115</td>
<td>12.8</td>
<td>3.7*</td>
<td>9.5*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mut vs.</td>
<td>81</td>
<td>1.2</td>
<td>1.8</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>WT</td>
<td>113</td>
<td>0</td>
<td>1.9</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mut</td>
<td>83</td>
<td>0</td>
<td>1.8</td>
<td>4.6</td>
</tr>
<tr>
<td>Siena et al. [44]</td>
<td>First-line treatment</td>
<td>FOLFOX + panitumumab vs. FOLFOX</td>
<td>WT = 656</td>
<td>55</td>
<td>9.6 (wt)*</td>
<td>7.3 (mut)</td>
<td>N.R.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mut = 440</td>
<td>48</td>
<td>8.0 (wt)</td>
<td>8.8 (mut)</td>
<td></td>
</tr>
<tr>
<td>Kohne et al. [45]</td>
<td>First-line treatment</td>
<td>FOLFIRI + panitumumab</td>
<td>WT</td>
<td>85</td>
<td>48</td>
<td>N.R.</td>
<td>N.R.</td>
</tr>
</tbody>
</table>
A number of MEK inhibitors such as AS703026, signal-regulated kinase (ERK) as a substrate (see Figure 1) known as MAP2K) is downstream of BRAF in the RAS-RAF-MEK signaling cascade of the EGFR and uses extracellular signal-regulated kinase (ERK) as a substrate (see Figure 1) [67]. A number of MEK inhibitors such as AS703026, AZD6244 and RO5068760 have been or currently are being investigated in phase 1 and 2 clinical trials [68, 69]. The development of several MEK inhibitors has been halted because of either very low response rates or due to ocular toxicity [70]. These agents have however shown substantial preclinical activity in tumor cell lines harboring the BRAF V600E gene mutation [71]. It has been established that KRAS has a number of downstream effectors that are not blocked by MEK inhibition, and indeed BRAF mutant cell lines were found to be more sensitive to MEK inhibitors than KRAS mutant cells [71]. It is imperative to be able to identify which patients are likely to respond to MEK inhibitors, and it appears that those with BRAF mutations are a good start. Given that KRAS signaling operates through a number of downstream effectors, those with KRAS mutations may require a combination of targeted agents. Preclinical evidence suggests that BRAF gene amplification is a mechanism of resistance to both MEK and BRAF inhibitors and a combination of these inhibitors may be a strategy to overcome this [72].

An additional EGFR pathway is the PTEN/PI3K/AKT pathway [phosphatase and tensin homologue gene (PTEN)]. PTEN encodes a phosphatase which uses phosphatidylinositol-3,4,5-trisphosphate (PIP-3) as a major substrate [73]. Loss of PTEN function leads to increased PIP-3 concentration, with resultant AKT hyperphosphorylation protecting tumor cells from apoptosis [73]. Roughly 60% of primary CRCs have a hyperphosphorylated AKT [74]. PTEN loss, activating mutations of phosphatidylinositol 3-kinase catalytic alpha polypeptide (PIK3CA) and activating mutations in KRAS/BRAF/MAPK confer resistance to apoptosis induced by cetuximab [75]. In patients with KRAS WT tumors treated with a cetuximab-based regimen, PTEN loss was associated with a significantly shorter OS [60]. Approximately one third of CRCs harbor activating somatic mutations in PIK3CA, and it has been reported that these mutations are predictive of lack of benefit from antiEGFR therapy [76]. Additional genetic alterations which could confer resistance to antiEGFR monoclonal antibodies include an inhibitor of PI3K signaling; coamplification of PAK4 (p-21-activated protein kinase) and AKT, which are downstream mediators of PI3K signaling; and amplification of IRS2 (insulin receptor substrate 2), which is an upstream activator of PI3K signaling [77, 78].

### 4. Strategies to Overcome Resistance

A number of approaches to the problem of resistance to antiEGFR monoclonal antibody therapy have been studied and are ongoing. Combining antiEGFR monoclonal antibodies with cytotoxic chemotherapy has already been discussed. Erlotinib and gefitinib, two oral small molecule EGFR inhibitors, are inactive by themselves [79, 80]. The combination of erlotinib with capecitabine and oxaliplatin in previously treated patients and the combination of gefitinib with FOLFOX were investigated in small phase II studies with favorable results, however randomized trials with chemotherapy alone as a control are needed [81–83]. Dual antiEGFR therapy with antiEGFR monoclonal antibodies plus antiEGFR TK inhibitors may overcome resistance to either drug alone. A 41% response rate was reported for the combination of cetuximab and erlotinib in patients with refractory disease, however this was limited to patients with KRAS and BRAF WT tumors [84].

EGFR and vascular endothelial growth factor (VEGF) have several signal transduction pathways in common, with preclinical data revealing that antiEGFR and antiVEGF drug combinations have synergistic activity [85]. The BOND-2 study randomized patients with irinotecan- and oxaliplatin-refractory but bevacizumab naïve disease to cetuximab and bevacizumab with or without irinotecan [21]. Response rates, TTP and OS favored the triple drug regimen, however, these results did not hold up in a subsequent study of this combination [21, 22]. Two subsequent randomized phase III trials have shown that combinations of antiEGFR...
monoclonal antibodies plus bevacizumab do not improve outcomes and can actually cause increased toxicity irrespective of KRAS mutational status. The PACCE trial evaluated panitumumab combined with oxaliplatin- or irinotecan-based chemotherapy plus bevacizumab. The dual monoclonal antibody arm was associated with increased toxicity and significantly shorter PFS in patients with both KRAS WT and mutant tumors [50]. Similar results were observed with the combination of cetuximab to a regimen containing capecitabine, oxaliplatin, and bevacizumab in the CAIRO2 trial [49].

Novel agents and combinations are being employed in an attempt to overcome anti-EGFR monoclonal antibody resistance. Motesanib, an oral inhibitor of VEGF, platelet derived growth factor (PDGF) and Kit receptors is being investigated with or without panitumumab in patients with refractory disease [86]. A number of inhibitors of the mutant BRAF kinase are in clinical development, as discussed above [87]. AMG 102 is an investigational monoclonal antibody against human hepatocyte growth factor (also known as cMET, of which overexpression correlates with cetuximab resistance) is being studied in combination with panitumumab in patients with metastatic CRC [88, 89].

5. Neoadjuvant and Adjuvant Therapy

Given the clinical benefit of anti-EGFR monoclonal antibodies in patients with metastatic disease, evaluation of these therapies as postoperative (adjuvant) treatment was warranted. In the adjuvant setting, eradication of micrometastatic disease is associated with increased cure rates. N0147 randomized 1760 patients with resected stage III KRAS WT colon cancer to FOLFOX with or without cetuximab [90]. Interim analysis led to premature closure of this trial after it was determined that no group of patients benefited from cetuximab [90]. Initially this trial enrolled patients regardless of KRAS mutational status, and among 658 patients with mutant KRAS, the addition of cetuximab to FOLFOX resulted in impaired disease-free survival (DFS) and a trend toward impaired OS [91].

In patients with rectal cancer, EGFR is a logical target in combination with neoadjuvant radiotherapy (RT). Retrospective analyses have demonstrated lower pathologic complete response (pCR) rates and shorter DFS in patients with rectal cancer expressing EGFR who were treated with neoadjuvant RT, suggesting that radiosensitivity might be increased by targeting the EGFR [92, 93]. Several phase I/II studies have investigated the combinations of cetuximab and chemoradiotherapy in the neoadjuvant setting for patients with rectal cancer. These studies have demonstrated that cetuximab could be safely combined with preoperative chemoradiotherapy but the pCR rates have been low (5–12%) [94–100]. In two of these studies [96, 99], subsequent analyses were done to correlate KRAS mutation status with response rate. Among patients with KRAS WT tumors, Bengala et al. reported a trend toward a greater rate of tumor regression (36.7% for KRAS WT versus 11% for KRAS mutant), however it did not reach statistical significance (P = .12) [101]. Debucquoy et al. also did not find a correlation between KRAS WT tumors and pathologic response to therapy [102]. To our knowledge, panitumumab has not been studied in combination with RT in patients with rectal cancer. Given the failure of anti-EGFR monoclonal antibodies to demonstrate a benefit in the adjuvant setting for stage III WT KRAS colon cancer, the value of further study of these agents for rectal cancer is doubtful.

Preclinically gefitinib has demonstrated improved radiosensitization [103]. Valentini et al. investigated the combination of gefitinib, continuous infusion 5-fluorouracil (5-FU) and pelvic RT in 41 patients with locally advanced rectal cancer and reported a pCR rate of 30%, however toxicity was an issue and further studies are necessary to establish the safety of this combination [104].

The effect of combined anti-EGFR and anti-VEGF therapy in combination with preoperative chemoradiotherapy for rectal cancer remains unknown, however given the negative results reported for combined EGFR and VEGF blockade in patients with metastatic CRC in combination with chemotherapy, studies investigating this avenue are unlikely [22, 49, 50]. Blaszkowsky et al. performed a small study investigating the combination of bevacizumab, erlotinib and 5-FU with RT in patients with locally advanced rectal cancer [105]. The regimen was found to be well-tolerated and highly active with a pCR rate of 47% and may deserve further investigation. However, the value of pCR as a surrogate for DFS and OS is uncertain.

6. Conclusion

Anti-EGFR monoclonal antibodies are among the standard treatment options for patients with metastatic CRC given their established efficacy. It is now clear that the benefit of anti-EGFR monoclonal antibodies is isolated to patients with KRAS WT tumors. It appears that KRAS mutational status is just the beginning of our understanding of the EGFR as an integral component of the biology of CRC. Given that only a subset of patients respond to anti-EGFR therapy, there is a need for better predictors to guide patient selection for such therapy. Several important components of the EGFR signaling pathway have been discovered, including BRAF, PTEN, AKT and PI3K, which deserve further study as predictors of response to existing treatments, or as targets of new interventions. The unexpected detrimental outcome associated with combined EGFR and VEGF blockade is a reminder of how much there is still to learn. New combinations and novel agents will continue to shed light on how to overcome resistance to inhibitors of the EGFR pathway, and hopefully new targets will be identified. Further study of how to employ our knowledge of EGFR pathway inhibitors to improve outcomes in the adjuvant and neoadjuvant setting is also warranted.

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Review Article

Gastrointestinal Mesenchymal Neoplasms other than Gastrointestinal Stromal Tumors: Focusing on Their Molecular Aspects

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Gastrointestinal (GI) mesenchymal tumors other than gastrointestinal stromal tumor (GIST) are rare neoplasms, but they often enter the differential diagnosis of more common GI lesions. Some of these mesenchymal tumors in the GI tract have well-understood molecular pathologic aspects, including desmoid tumors, inflammatory myofibroblastic tumor (IMT), clear cell sarcoma (CCS), inflammatory fibroid polyp (IFP), and synovial sarcoma (SS). Molecular pathology is fast becoming a mainstream focus in laboratories because it aids in the precise classification of tumors, may be prognostic, and may help predict response to therapy. The following review is not intended as an exhaustive summary of all mesenchymal tumors that have been reported to involve the GI tract, but instead will highlight the current knowledge of the most important non-GIST GI mesenchymal neoplasms, focusing on those tumors with well-characterized molecular pathology and how the molecular pathologic features impact current diagnostic, therapeutic, and prognostic standards.

1. Introduction

Gastrointestinal (GI) mesenchymal tumors are rare, and the molecular pathology of many of these tumors is unknown or poorly characterized. However, some mesenchymal tumors in the GI tract have well-understood molecular pathologic aspects. Molecular pathology is fast becoming a mainstream focus in laboratories because it aids in the precise classification of tumors, may be prognostic, and may help predict response to therapy. A search of the catalogue of somatic mutations in cancer (COSMIC) database for all mesenchymal tumors in the tubular GI tract and adjacent soft tissues, including esophagus, stomach, small intestine, large intestine, peritoneum, and retroperitoneum reveals meaningful data on three tumor types: gastrointestinal stromal tumor (GIST), inflammatory fibroid polyp (IFP), and desmoid tumors. Other mesenchymal tumors that occur in or around the tubular GI tract with well-characterized molecular pathologic features include synovial sarcoma (SS), inflammatory myofibroblastic tumor (IMT), and clear cell sarcoma (CCS); these tumors are characterized by translocations rather than mutations. The following paper is not intended as an exhaustive summary of all mesenchymal tumors that have been reported to involve the GI tract, but instead will highlight the current knowledge of the most important non-GIST GI mesenchymal neoplasms, focusing on those tumors with well-characterized molecular pathology.

2. Intraabdominal Desmoid Tumors

Intraabdominal desmoid tumors arise in the mesentery or retroperitoneum, predominantly in young patients. Approximately 10% of desmoids occur in patients with familial adenomatous polyposis [1] as one of the extracolonic manifestations of Gardner syndrome. Desmoids do not metastasize, but they often recur locally [1, 2]. The histologic features of desmoids are quite characteristic. In particular, these tumors show low to moderate cellularity and are composed of uniform spindle cells with a small,
nucleolus arranged in long, sweeping fascicles (Figure 1) [1]. The vasculature shows small arteries with accompanying veins and a mild perivascular lymphoid infiltrate. The associated stroma is quite collagenous. Mitotic figures may be 10/50 high power field (HPF) or more [3,4], but these tumors lack other histologic features of malignancy such as dense cellularity, cytologic atypia, or atypical mitotic figures.

Important differential diagnostic considerations include sclerosing mesenteritis, which does not invade bowel wall [5] and IgG4-related sclerosing disorders, which are rich in IgG4 plasma cells [6].

Most desmoid tumors arise via perturbations within the Wnt signaling pathway (Figure 2). In FAP, desmoid tumors arise from mutations in the adenomatous polyposis coli (APC) gene, located on chromosome 5q21-22, which encodes a tumor suppressor protein, although its function may be more complex than simply a tumor suppressor [7]. Inactivation of APC leads to nuclear accumulation of β-catenin, causing increased transcription and cell proliferation [1]. Most APC mutations associated with desmoid tumors are found 3’ to codon 1400 [8,9]. Some sporadic desmoid tumors also arise from APC inactivation [10] but most (>80%) are APC wild type with activating mutations of the CTNNB1 gene, which is located on chromosome 3p22-p21.3. CTNNB1 encodes β-catenin [11]. Mutations almost exclusively occur at codons 41 and 45 in exon 3 of CTNNB1 [12]; mutational analysis of CTNNB1 is usually not necessary in typical cases of desmoids tumors but can be helpful in unusual cases as well as helpful in distinguishing recurrent desmoid from scar. It remains to be determined whether particular CTNNB1 mutations help predict local recurrence after surgical resection. One report [13] found that tumors harboring S45F mutations in exon 3 of CTNNB1 had significantly poorer disease-free survival compared to wild-type tumors or codon 41 mutants, whereas another report showed no significant differences in recurrence-free survival among CTNNB1 mutants but did show worse outcome among all mutants compared to wild-type tumors [14].

Regardless of inciting molecular event, the final common pathway is accumulation of β-catenin protein within the nuclei of the tumor cells, and although most desmoids do not require immunohistochemistry for diagnosis, nuclear staining of with β-catenin characterizes >90% of desmoid tumors [1]. In our experience, β-catenin immunohistochemistry is less reliable than the literature reports, particularly in needle biopsies, and molecular testing for mutations in the β-catenin gene may be more reliable. Immunoreactivity with CD117 has been reported in the literature [4], but this is generally considered an anomaly; when appropriate titers and antigen retrieval methods are used, desmoids show no CD117 positivity [15–18]. Although some investigators have reported response to imatinib [19], others have found that imatinib showed the lowest response rates in comparison to other forms of systemic therapy [20]. Furthermore, members of the PDGFR family are expressed in the majority of desmoids, but this expression does not correlate with response to imatinib. Since no clear target is present on desmoid tumors, imatinib therapy is controversial at best. Surgery remains the mainstay of treatment, but surgical resection is often incomplete because of the infiltrative growth of desmoid tumors, with recurrences up to 38% [13]. In these patients, systemic therapies such as anti-inflammatory, hormonal, cytotoxic chemotherapy, and radiation are considered. In patients with FAP, surgery such as ileal pouch procedures may trigger desmoid tumor formation [1].

3. Inflammatory Myofibroblastic Tumor (IMT)

IMTs are a heterogenous group of spindle cell proliferations with admixed lymphocytes and plasma cells that tend to occur in children and young adults. The omentum and mesentery are the most common extra-pulmonary sites for IMT [21,22], and these tumors may have a more aggressive biologic behavior, with more frequent recurrences [23]. Most IMTs harbor a heterogenous microscopic appearance and may contain any combination of nodular fasciitis-like areas, compact spindle cell proliferations, or paucicellular scar-like areas, making them a challenging diagnostic entity. In all tumors, inflammatory cells are a distinctive feature, and the infiltrate is often rich in plasma cells (Figure 3) [22].

Some authorities have differentiated IMTs from reactive pseudosarcomatous processes and other neoplasms based on ALK immunoreactivity or evidence of the ALK translocation, but many consider ALK-negative IMTs a valid diagnostic category. Tumors with ALK rearrangements are associated with younger age and strongly correlate with ALK protein expression detected by immunohistochemistry in some labs, but in other labs ALK immunohistochemistry is nearly always negative. Cook et al. found that 12/20 (60%) of cases expressed ALK by immunohistochemistry [24]. In fact, the literature reports that only about half of IMT harbor an ALK translocation, and these may behave more indolently than their ALK-negative counterparts [23], although this may not be the case for all ALK-rearranged tumors. For example, a very recent series described 11 intra-abdominal tumors characterized by epithelioid morphology, abundant

Figure 1: Photomicrograph of H&E-stained section from a desmoid tumor. Note the moderately cellular sweeping fascicles of bland spindle cells. The lower left inset contains a high-power photomicrograph of the same tumor to demonstrate the characteristic pinpoint nucleoli and collagenous stroma of desmoids.
Figure 2: Schematic of Wnt signaling pathway. In demoid tumors, mutations are usually found in the APC gene or CTNNB1 gene, which encodes β-catenin. Regardless of the primary defect, the end result is nuclear accumulation of β-catenin, which fails to undergo cytoplasmic degradation.

Figure 3: Photomicrograph of H&E-stained section from an inflammatory myofibroblastic tumor. The tumor is relatively cellular and composed of a mixture of plump spindle cells and inflammatory cells, particularly lymphocytes and plasma cells.

Figure 4: Photomicrograph of FISH break apart probe that targets the ALK gene. The normal chromosome (lower middle) shows a green signal and red signal in close proximity, whereas the green and red signals are far apart in the left aspect of the photomicrograph, confirming 1 copy of the ALK translocation.

myxoid stroma, and frequent neutrophils [25]. These tumors demonstrated aggressive behavior with rapid local recurrence, death in 5, and metastasis in 2. All 11 showed nuclear membrane or peri-nuclear ALK immunoreactivity. Nine tumors showed ALK translocation, and 3 tested showed ALK/RANBP2 rearrangement.

Tumors other than IMTs have been recognized to demonstrate immunohistochemical expression of ALK, so genetic confirmation of the ALK translocation may be needed in problematic cases. At our institution, we find break-apart FISH testing to be quite useful in this setting (Figure 4). It should be noted, however, that a very recent report of response to the ALK inhibitor crizotinib in one patient with confirmed ALK translocation and no response in an IMT without ALK rearrangement [26] provides preliminary justification for FISH testing on all suspected IMTs. Secondary resistance to crizotinib in an IMT with ALK translocation (ALK/RANBP2) has been recently documented in another patient. This resistance was suspected to occur via the neuroblastoma-associated F1174L ALK mutation that has been well studied in neuroblastomas as a mechanism of resistance to some ALK inhibitors [27].

ALK perturbations in IMTs occur via fusion of the C-terminal kinase domain of anaplastic lymphoma kinase (ALK) gene located on 2p23. The ALK gene encodes a tyrosine kinase receptor that is normally only expressed in
the developing nervous system [28]. In IMTs, the ALK gene fusion partner is most commonly tropomyosin 3 (TPM3/ALK) or tropomyosin 4 (TPM4/ALK), leading to constitutive activation. Other reported fusion partners include CLTC, ATIC, RANBP2, CARS, and SEC31L1 (Table 1). ALK along with its fusion partner tends to localize to the cytoplasm, but ALK/RANBP2 localizes to the nuclear membrane [29]. ALK function is poorly characterized at this time, so it is difficult to postulate the exact mechanism of oncogenesis; nevertheless, these fusions clearly lead to a survival and growth advantage to the cells harboring the translocation.

### 4. Inflammatory Fibroid Polyp (IFP)

IFPs are most often encountered in the stomach, usually presenting in the antrum, but are found throughout the GI tract. IFPs tend to arise within the submucosa and frequently extend into the overlying mucosa. These tumors are composed of bland spindle cells that often form perivascular cuffs (Figure 5). The tumor cells are embedded in a distinctive, granulation-type or fibromyxoid stroma with abundant capillary-type vessels. Characteristically, IFPs show a prominent eosinophilic infiltrate, but other inflammatory cells such as lymphocytes, mast cells, plasma cells, and histiocytes are encountered [1]. Given their gastrointestinal location, overlapping molecular features, and characteristic CD34 immunoreactivity, IFPs may be confused with GISTs [17]. Importantly, IFPs do not express other GIST-specific markers such as KIT or DOG-1 [36]. A few patients with germline PDGFRA mutations have been reported in the literature. The reports tend to consider these patients within the spectrum of familial GIST, but they present with a variety of tumors including GIST, GI neurofibromas, lipomas, and IFP-like polyps, some of which demonstrate a lipomatous stroma [12, 37, 38].

IFPs are rare benign tumors of uncertain histogenesis and were considered reactive processes, but recently, mutations in platelet-derived growth factor receptor alpha (PDGFRα) were described in IFPs located in the stomach and small bowel [39]. In the stomach, 16/23 (70%) IFPs harbored activating mutations of PDGFRα [40]. Six of the mutations were located in exon 12, and 10 were located in exon 18. In the small bowel series, 33/60 (55%) harbored mutations in PDGFRα, 31 of which were located in exon 12, and 2 were in exon 18 [41]. Most of these mutations had been previously described in GIST. Extrapolating from the GIST literature, 41 of the 49 mutations detected in these series have been shown to cause at least in vitro ligand-independent activation. In addition, 100% of the stomach IFPs and 95% of small bowel IFPs showed expression of PDGFRA detected by immunohistochemistry, leading to the hypothesis that the cell of origin is a PDGFRA-positive mesenchymal cell [41].

PDGFRA is a receptor tyrosine kinase that is highly homologous to KIT [41]. Activation is normally ligand-dependent, but activating mutations cause constitutive activation (Figure 6). Ligand binding can cause homodimerization with another PDGFRA or hetero-dimerization with a PDGFRA-beta. PDGFRα’s interaction with several signaling pathways such as RAS/MAPK, PI3K, and JAK/STAT.
allows for the acquisition of numerous tumorigenic cell functions such as cell growth, migration, inhibition of apoptosis, and angiogenesis [42] when the receptor is constantly activated. The utility of PDGFRA mutation testing to confirm the diagnosis of IFP is minimal, but it is important to realize that not all PDGFRA-mutated mesenchymal neoplasms in the GI tract are GISTs.

5. Clear Cell Sarcoma (CCS)

Primary GI CCSs are extremely rare, with only about 20 total cases reported in the literature. Age range is quite variable, with the youngest diagnosed at age 13 years and the oldest at 85 [43]. The small bowel is most frequently involved, but reports of gastric, colonic, and mesenteric tumors also exist. These tumors are often metastatic to peritoneum, lymph nodes, or liver at presentation and are at significant risk for local recurrence after surgery [43]. Morphologically and immunophenotypically, there appear to be two CCS-like malignancies that occur in the GI tract.

The first type of GI CCS constitutes those tumors that are morphologically, ultrastructurally, and immunohistochemically indistinguishable from CCSs of soft tissue. CCSs are typically composed of nests and fascicles of pale spindled to epithelioid cells, separated by delicate fibrous septa, forming a lobulated or organoid growth pattern (Figure 7). Cellular pleomorphism is typically uncommon, and multinucleated giant cells may be present. Nucleoli are typically prominent. Immunohistochemically, these tumors are indistinguishable from melanoma, being S-100 protein as well as melanocytic markers such as HMB-45 and Melan-A positive in the majority of cases [43]. In the soft tissue, these tumors are often referred to as “melanoma of soft parts,” because of their morphologic and immunohistochemical resemblance to malignant melanoma. Unfortunately, immunohistochemistry is of no help distinguishing the two malignancies, but the characteristic growth pattern and bland cytologic features with pale cytoplasm are useful clues to the diagnosis. As discussed below, rearrangements in the EWSR1 gene occur in CCS, not melanoma, which can be invaluable in separating CCS from melanoma [44].

The second tumor type encountered in the GI tract is characterized by mostly epithelioid cells with pale cytoplasm without the characteristic tumor cell nesting or compartmentalization of soft tissue CCS. Osteoclast-like multinucleate giant cells are by definition admixed with the tumor cells such that the tumor has been described as “Osteoclast-rich tumor of the GI tract resembling CCS of soft parts.” The tumor cells reveal S-100 protein expression but lack any ultrastructural or immunohistochemical evidence of melanocytic differentiation. Other important morphologic differences with conventional CCS of soft tissue include indistinct nucleoli, more mitotic activity, and prominent cellular pleomorphism [43].

More than 90% of CCS of soft tissue are associated with the reciprocal translocation t(12;22)(q13;q12), which results in fusion of the EWSR1 gene and the ATFI gene. This translocation links the N-terminal domain of EWSR1 to the basic leucine zipper of ATFI. [43] Four fusion transcripts (Table 2) have been described in soft tissue tumors, the most common, or type 1, is a fusion of exon 8 of EWSR1 and exon 4 of ATFI [45]. This translocation thus far has been the only described EWSR1/ATFI translocation in CCS of the GI tract. Another EWSR1 translocation, EWSR1/CREB1, representing the t(2;22)(q34;q12) translocation, recently has been identified in a subset of GI and soft tissue CCS [46–48].

CREB1 and ATFI belong to the basic leucine zipper superfamily of basic leucine zipper transcription factors. In normal melanocytes, CREB and ATFI are involved in driving melanocytic differentiation. Both EWSR1/ATFI and EWSR1/CREB1 fusion transcripts retain the basic leucine zipper domain, which mediates DNA binding and dimerization. In EWSR1/ATFI translocations, the activating domain of EWSR1 replaces the kinase inducible domain of ATFI, and the protein product has been shown to bind to microphthalmia-associated transcription factor, which in turn activates melanocyte stimulating hormone [29]. Overexpression of CREB contributes to metastatic potential in melanoma cells and is oncogenic in myeloid lines [46]. Genotype-phenotype correlation is imperfect in these GI tract CCS-like tumors such that either the EWSR1/ATFI or EWSR1/CREB1 may occur in either tumor morphology described above [46].

Due to the rarity of CCS cases occurring in the GI tract, the first hurdle is considering the diagnosis. RT-PCR or FISH analyses are critical to the diagnosis of CCS, given its morphologic and immunophenotypic overlap with melanoma (Figure 8). It remains to be determined whether these

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Table 2: Summary of various EWSR1 fusions in CCS [45, 46].

<table>
<thead>
<tr>
<th>EWSR1/ATFI transcript</th>
<th>Relative frequency</th>
<th>GI tract</th>
</tr>
</thead>
<tbody>
<tr>
<td>EWSR1 exon 8/ATFI exon 4</td>
<td>common</td>
<td>Yes</td>
</tr>
<tr>
<td>EWSR1 exon 7/ATFI exon 5</td>
<td>common</td>
<td>No</td>
</tr>
<tr>
<td>EWSR1 exon 10/ATFI exon 5</td>
<td>uncommon</td>
<td>No</td>
</tr>
<tr>
<td>EWSR1 exon 7 ATFI exon 7</td>
<td>uncommon</td>
<td>No</td>
</tr>
<tr>
<td>EWSR1 exon 7/CREB1 exon 7</td>
<td>uncommon</td>
<td>Yes</td>
</tr>
</tbody>
</table>

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Figure 7: Photomicrograph of H&E-stained section from a conventional clear cell sarcoma. The cells are relatively bland with variably prominent nucleoli, low mitotic activity, and pale to clear cytoplasm. Fibrous bands illicit a compartmentalized appearance.
S-100 protein positive tumors harboring EWSR1 translocations represent a clinical, morphologic, immunophenotype, and genetic spectrum of one tumor [46] or are two distinct tumors [43].

6. Synovial Sarcoma (SS)

Primary GI SSs are quite uncommon. The Armed Forces Institute of Pathology (AFIP) reported a series of 10 SSs after undertaking a 30-year review of stomach mesenchymal neoplasms from 3 large centers. Prior to this series, there were 7 total reports of SS, 6 of which involved the esophagus, 1 involved the stomach, and all were biphasic. The AFIP series found 4 males and 6 females with an age range of 29–68 years. Two of the 8 patients with adequate followup died of their disease and two more showed local recurrence. Most tumors form a plaque-like mucosal mass and show uniform spindle cells in a haphazard arrangement with a collagenous background. Calcification or osseous metaplasia may be present. Nine of the 10 cases were of the monophasic type with one showing a poorly differentiated round cell component with high mitotic activity. The final case was biphasic, demonstrating a mixture of epithelial and spindled components. Mitotic activity is variable and can be greater than 50 per 10 HPF. All 7 tumors tested in the AFIP series showed the character SYT/SSX fusion transcripts (3 with SYT/SSX1 and 4 with SYT/SSX2) [49].

The characteristic molecular alteration in SS is the t(X;18)(p11;q11) translocation, which usually fuses SYT on chromosome 18 with SSX1 or SSX2 on the X chromosome. Other less common fusion partners include SSX4, also located on the X chromosome. An SYT homolog, SS18LI, located on chromosome 20 also has been described as a fusion partner of SSX1 in SS [50]. The functions of these gene products are unknown, and the oncogenic effects of the SYT/SSX fusion protein are unclear. Other genetic events may be necessary for sarcomagenesis and other molecular alterations have been described, including ERBB2 expression, IGF2 upregulation, CD44 repression, PTEN inactivation, and mutations associated with the wnt pathway [28]. Break-apart FISH probes are invaluable at our institution in confirming the diagnosis of SS, particularly in monophasic fibrous types, but PCR-based assays are used effectively at other institutions [50].

7. Smooth Muscle Tumors

Leiomyomas of the GI tract show a male predominance and are most common in the colon and rectum, where they actually outnumber GIST [1]. The vast majority of GI leiomyomas are benign smooth muscle proliferations arising from the muscularis mucosae, often giving a polypoid endoscopic appearance (Figure 9) [51]. Rarely, cytologic atypia (“symplastic” leiomyoma) or even more rarely, mitotic activity may occur [18]. The second most common location for GI leiomyoma is in the distal esophagus, where they also outnumber GIST [51]. Esophageal leiomyomas usually are intramural in location, arising in the muscularis propria. Esophageal leiomyomas often show undulating borders and can range in size from <1 mm to >10 cm [1]. Various X chromosomal abnormalities, including collagen type IV alpha 5 and alpha 6, have been described in esophageal leiomyomas, which potentially accounts for the male predominance in these neoplasms [18]. GI leiomyomas may be confused with GISTs, and immunohistochemistry in this differential diagnosis can be quite helpful. Leiomyomas are invariably strongly positive for desmin, but are negative for CD117. One must not confuse CD117-positive mast cells that may be seen in between smooth muscles cells from true CD117 immunoreactivity in the smooth muscle cells [52].

GI leiomyosarcomas are quite rare [18, 53]. They are generally large masses and are characterized by cytologic atypia, high mitotic rate, and necrosis. Extra-intestinal leiomyosarcoma may show complex cytogenetic abnormalities [29] and have been recently shown to have loss of chromosomes 10q...
and 13q along with amplification of 17p13.1 to 11.2 [54], but the molecular pathology of GI leiomyosarcoma is unknown.

8. Schwannoma

Schwannomas are most commonly encountered in the stomach [55] but are found throughout the GI tract [56, 57]. They may present as polyps or intramural masses. Their histologic appearance is often somewhat different than their extra-GI counterparts (Figure 10). GI schwannomas usually lack well-developed nuclear palisading, hyalinized vessel walls, or a capsule. Only rarely are the characteristic Antoni A and B patterns well developed, and Verocay bodies usually are not encountered. Distinguishing most schwannomas requires observation of the typical “wavy” nuclei with tapered ends; however, a minority may demonstrate an epitheliod morphology [57]. Regardless of morphology, all tumors demonstrate diffuse S-100 protein immunoreactivity while lacking CD117 positivity. Another characteristic feature of GI schwannomas is a prominent lymphoid infiltrate at the tumor periphery. GI schwannomas are benign and do not seem to occur in the setting of NF2 [1]. In addition, GI schwannomas show no NF2 mutations and only rare LOH of ch. 22q [56], which is different than their soft tissue counterparts. Rare examples tested for KIT mutations have been wild type [58].

9. Mesenchymal Polyps

A variety of mesenchymal lesions present as incidentally identified polyps during routine endoscopic procedures (usually colonoscopy), including perineuriomas/fibroblastic polyps [59], muscularis mucosae leiomyomas [51], Schwann cell “hamartomas,” [60], granular cell tumors [61], elastofibromatous polyps [62], and ganglioneuromas [63]. Colonic perineuriomas/fibroblastic polyps are often associated with a hyperplastic polyp-like component with serrated epithelial crypts. A recent study reported BRAF mutations in 63% of the epithelial component of these polyps. The authors concluded that the polyps are true mixed epithelial-mesenchymal neoplasms, but there remains no conclusive evidence that the spindle cell proliferation is truly neoplastic [64]. Ganglioneuromas are benign lesions composed of ganglion cells, nerve fibers, and Schwann cells [60] that often arise in the setting of inherited tumor syndromes, including Cowden syndrome (PTEN mutations), MEN 2B, and NF1 [60], among others. Solitary ganglioneuromas are not considered to be a marker of an inherited syndrome. Aside from syndrome-associated ganglioneuromas, the molecular pathologic aspects of these benign polypoid mesenchymal lesions are uncharacterized.

10. Adipocytic Tumors

Lipomas are most common in the right colon and usually are identified as small intramural polypoid lesions. They are usually centered within the submucosa and composed of mature appearing adipocytes that are relatively uniform in size and lack cytologic atypia (Figure 11). These lesions are often endoscopically suspected after eliciting the “pillow sign” with closed biopsy forceps [65]. No molecular characterization of GI lipomas exists; conventional soft tissue lipomas exhibit abnormal karyotypes in about 60% of cases, most commonly involving rearrangement of chromosome 12q13~15 encompassing the chromatin remodeling gene HGMA2 [66].

Primary GI liposarcomas are exceptionally rare tumors. Most liposarcomas that involve the gut arise within the retroperitoneum, and this is one of the more common causes of a sarcoma presenting as a GI mass. Typical well-differentiated liposarcomas are fatty tumors that demonstrate large, atypical cells embedded within fibrous septa or between the fat cells, but other morphologies such as inflammatory or sclerosing exist. Conventional liposarcoma may de-differentiate and at least partially loose their typical well-differentiated component. About 80% of well-differentiated
liposarcomas are characterized by ring or giant marker chromosomes derived from chromosome 12q13~q15, including MDM2, HGMA2, and other genes [67]. When more sensitive methods such as FISH, PCR, or immunohistochemistry are employed, >95% of tumors show MDM2 amplification [68, 69]. Therefore, assessing for amplification of MDM2 by FISH or immunohistochemistry is a powerful tool in supporting the diagnosis liposarcoma secondarily involving the GI tract.

11. Glomus Tumors

These tumors are morphologically similar to their counterparts that are most commonly encountered in the distal extremities. The vast majority of GI glomus tumors have been documented to occur in the stomach with fewer occurring in the intestines and <150 cases have been reported in the English literature [70]. Glomus tumors are composed of a proliferation of sharply demarcated modified smooth muscle cells, which are often arranged around dilated staghorn vessels. The cells contain a round nucleus and pale cytoplasm and generally low mitotic activity. Focal atypia and vascular invasion reportedly are common. In the AFIP series of 32 gastric glomus tumors, none of the 5 tumors tested for mutations in exons 9 or 11 of the KIT gene showed a mutation [70]. Otherwise, the molecular pathology of glomus tumors is unknown.

12. Conclusion

Primary mesenchymal tumors of the GI tract are rare, but like their soft tissue counterparts, molecular pathology often plays a critical role in the work-up of these tumors. Molecular pathology plays a major role in the diagnosis of these tumors, particularly in clear cell sarcoma, inflammatory myofibroblastic tumor, synovial sarcoma, and liposarcoma, among others. Powerful prognostic data also is emerging such as in inflammatory myofibroblastic tumor, synovial sarcoma, and liposarcoma, among others. Powerful prognostic data also is emerging such as in inflammatory myofibroblastic tumor with ALK rearrangements and possibly CTNNB1 mutations in desmoids. The histological features of desmoids in the AFIP series of 32 gastric glomus tumors, none of the 5 tumors tested for mutations in exons 9 or 11 of the KIT gene showed a mutation [70]. Otherwise, the molecular pathology of glomus tumors is unknown.

References


EGFR Signaling in Colorectal Carcinoma

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The epidermal growth factor receptor (EGFR) and its downstream signaling pathways are involved in the development and progression of several human tumors, including colorectal cancer. Much attention has been given to the EGFR pathway as of lately because both EGFR and some downstream components serve as targets for anticancer therapy. In addition to playing a critical role in targeted therapy, alterations in this pathway can have prognostic implications. The EGFR pathway and its impact on colorectal carcinogenesis and prognosis are the emphasis of this paper. Since prognosis is tightly related to response to various therapies, the predictive value of the components of this pathway will be briefly discussed, but this is not the focus of this paper.

1. Introduction

The epidermal growth factor receptor (EGFR) and its downstream signaling pathways regulate key cellular events that drive the progression of many neoplasms. EGFR is expressed in a variety of human tumors, including gliomas and carcinomas of the lung, colon, head and neck, pancreas, breast, ovary, bladder, and kidney. Mutations, gene amplification, and protein overexpression of various elements of this pathway not only contribute to carcinogenesis but also impact prognosis and provide specific targets for therapeutic intervention. The importance of EGFR and its signaling pathway in colorectal carcinogenesis is the topic of this paper. Since prognosis is tightly related to response to various therapies, the predictive value of the components of this pathway will be discussed, but only briefly. There is another paper in this series, “Impact of KRas mutations on management of colorectal cancer” by Sullivan and Kozuch, which provides an in-depth review of the predictive value of KRas and other members of the EGFR signaling pathway.

2. EGFR and the EGFR Signaling Pathway

EGFR is a 170-kDa transmembrane tyrosine kinase receptor that belongs to the ErbB family of cell membrane receptors. In addition to EGFR (also known as HER1 and ErbB-1), other receptors in this family include HER2/c-neu (ErbB-2), Her 3 (ErbB-3), and Her 4 (ErbB-4). All of these receptors contain an extracellular ligand-binding region, a single membrane-spanning region, and a cytoplasmic tyrosine-kinase-containing domain.

In normal cells, the EGFR signaling cascade begins with ligand activation of EGFR (Figure 1). Up to eleven ligands can bind the ErbB family of receptors, including EGF and transforming growth factoralpha [1]. Ligand binding induces dimerization of the receptor with formation of homodimers and heterodimers, which leads to the activation of tyrosine kinase. The intracellular tyrosine kinase residues then become autophosphorylated, inducing activation of multiple signal transduction pathways. Two main intracellular pathways activated by EGFR are the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase- (PI3K-) protein kinase B (AKT) pathway. These pathways lead to the activation of various transcription factors that then impact cellular responses such as proliferation, migration, differentiation, and apoptosis [2].

Signaling through the EGFR pathway is a complex process that requires tight regulation [2]. The first level of complexity is encountered at the receptor level, where
Figure 1: EGFR signaling pathway. Ligand binding induces dimerization and activates the EGFR. Subsequent autophosphorylation of tyrosine residues activates downstream signaling. In the Ras-Raf-MEK-MAPK, one axis of the EGFR signaling cascade, an adaptor protein complex composed of growth factor receptor-bound protein 2 adapter protein (Grb2), which harbors a tyrosine phosphate-docking site, and son of sevenless (SOS), a Ras GDP/GTP exchange factor, then activates the Ras GTPase. After activation, Ras (i.e., KRas) recruits and activates the serine protein Raf (i.e., B-Raf), and subsequent phosphorylation and activation of MEK and then MAPK occurs, resulting in activation of transcription factors in the cell nucleus. The Ras-Raf-MAPK signaling pathway is thought to control cell growth, differentiation, and survival (apoptosis). The other axis of the EGFR signaling cascade that is important in colorectal carcinogenesis is the PI3K-AKT pathway. Once the EGFR tyrosine residues are phosphorylated, PI3K is translocated to the cell membrane and binds to tyrosine phosphate (through its adaptor subunit p85) which triggers the PI3K catalytic subunit p110 to produce phosphatidylinositol-3,4,5-triphosphate (PIP3). PI3K then promotes AKT activation. Activated AKT (p-AKT), present within the cytoplasm, then activates various targets that result in cell growth, proliferation, and survival (paralleling the Ras-Raf-MEK-MAPK signaling pathway). Importantly, these two axes are closely related and have some overlap. For example, the p110 subunit of PI3K can also be activated via interaction with Ras. Of note, phosphatase with tensin homology (PTEN) is a phosphatase that converts PIP3 back to phosphatidylinositol (4, 5) bisphosphate (PIP2), thereby negatively regulating the PI3K-AKT pathway.

multiple ligands are shared and lateral signaling occurs between members of the ErbB family. Then there are positive and negative feedback loops built into the pathways and differential activation of transcription factors, depending upon the cell type. When this tightly regulated system goes awry, it can contribute to malignant transformation and tumor progression through increased cell proliferation, prolonged survival, angiogenesis, antiapoptosis, invasion, and metastasis [3, 4].

3. The EGFR Pathway and Colorectal Carcinogenesis (Table 1)

3.1. EGFR Protein Expression. EGFR expression (or overexpression), typically determined by immunohistochemistry, has been found to be associated with tumor progression and poor survival in various malignancies, such as carcinomas of the head and neck [5]. However, the significance of EGFR protein expression is controversial in other tumors, such as lung carcinomas [6]. Although EGFR has been reported to be overexpressed in anywhere from 25% to 82% of colorectal cancers [4], some recent studies report protein overexpression (defined as 2+ and/or 3+ staining or in >50% of cells) in 35 to 49% of cases [7–9]. However, the clinical significance of EGFR overexpression in colorectal cancer is uncertain. While one study of 249 colorectal cancers demonstrated an association of EGFR overexpression with tumor grade (poor differentiation) ($P = .014$) [8], another group found no association with grade in 134 tumors [9]. Similarly, some studies have found an association between EGFR overexpression (defined as 2+ or 3+ intensity) and reduced survival [7, 9], while others have not [4].

Due to the known expression of EGFR in colorectal cancer, a phase II trial of cetuximab, an anti-EGFR monoclonal antibody, in patients with refractory EGFR-positive
Table 1: Components of the EGFR signaling pathway important in colorectal cancer.

<table>
<thead>
<tr>
<th>Component (gene/protein)</th>
<th>Protein function</th>
<th>Defect in CRC</th>
<th>Frequency</th>
<th>Prognostic</th>
<th>Impact (to anti-EGFR therapy)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EGFR/EGFR</strong></td>
<td>Transmembrane tyrosine kinase receptor</td>
<td>Protein expression Mutation Increased copy number</td>
<td>25–90% Rare 0–50%*</td>
<td>Controversial Unknown</td>
<td>No correlation Unknown</td>
</tr>
<tr>
<td><strong>KRas/KRas</strong></td>
<td>GDP-/GTP-binding protein; facilitates ligand-dependent signaling</td>
<td>Activating mutation (codons 12, 13, 61, 146); leads to activation of MAPK pathway</td>
<td>30–40%</td>
<td>Controversial</td>
<td>No response (if KRas is mutated)</td>
</tr>
<tr>
<td><strong>BRAF/B-Raf</strong></td>
<td>Serine-threonine protein kinase downstream of KRas</td>
<td>Activating mutation (V600E)</td>
<td>5–12%</td>
<td>Poor prognosis in MSS tumors</td>
<td>No response (if BRAF is mutated)</td>
</tr>
<tr>
<td><strong>PIK3CA/PI3K</strong></td>
<td>A key signal transducer in the PI3K-AKT pathway</td>
<td>Activating mutation (exons 9 and 20)</td>
<td>14–18%</td>
<td>Poor prognosis in KRas wt tumors</td>
<td>No response (if exon 20 is mutated)</td>
</tr>
<tr>
<td><strong>PTEN/PTEN</strong></td>
<td>A protein tyrosine phosphatase enzyme; inactivates PI3K pathway</td>
<td>Loss of protein expression; mutation; LOH</td>
<td>13–19%</td>
<td>Poor prognosis in KRas wt tumors</td>
<td>No response (possibly)</td>
</tr>
</tbody>
</table>

CRC: colorectal cancer; LOH: loss of heterozygosity; wt: wild-type.

*Low % for high (>10 copies) amplification; higher % for low number of copies (3–5 copies).

(assessed by immunohistochemistry) colorectal cancer was undertaken [10]. The results of this trial, reported in 2004, were promising. It was soon discovered, however, that there was no correlation between EGFR expression in the tumor and response to therapy [11, 12]. In the study by Chung et al., four of 16 (25%) patients with EGFR-negative tumors who received cetuximab-plus-irinotecan therapy achieved a partial response with a greater than 50% reduction in the size of measurable lesions [11]. This response rate is nearly identical to the 23% response rate seen in a separate cetuximab-plus-irinotecan clinical trial in EGFR-positive patients [12]. As a result, cetuximab is now administered as indicated without the need for EGFR testing.

The wide range of EGFR expression in colorectal cancer reported in the literature, as well as the uncertain significance of EGFR expression as a prognostic indicator, may be related to the methodology used to detect EGFR. Most studies use immunohistochemistry to detect EGFR expression in colorectal cancers. As demonstrated by the experience of HER2 expression in breast cancer, immunohistochemistry is highly dependent on the antibody clone that is used, staining protocols, selection of scoring methods, and selection of cutoff values. Until a standard method of EGFR staining and reporting is adopted, the significance of EGFR protein expression in colorectal cancer remains controversial.

3.2. EGFR Mutations, Gene Amplification, and Copy Number.

Mutations affecting the extracellular domain of EGFR, often accompanied by gene amplification, are frequent in glioblastomas [13], while mutations in the tyrosine kinase domain of EGFR, also frequently associated with increased EGFR gene copy numbers, are clinically relevant in lung adenocarcinoma [6, 14–16]. Unlike lung cancer and other tumors, EGFR gene mutations are uncommon in colorectal cancers [17, 18].

The significance of EGFR gene amplification/increased EGFR copy number is more difficult to summarize. Some studies report that EGFR gene amplification (assessed by in situ hybridization methods) is uncommon in colorectal cancer [19, 20]. In contrast, in recent studies on chemotherapy-resistant colon cancers, it appears that modest increases in copy number (three- to fivefold) are present in up to 50% of cases [21]. It appears, however, that increased EGFR protein expression does not always translate into increased EGFR gene dosage [19, 21, 22]. For example, a study by Shia et al. found that only a small fraction (17% of 124 or 14%) of EGFR-positive (defined as 1+, 2+, or 3+) colorectal carcinomas detected by immunohistochemistry were associated with EGFR gene amplification (defined as >5 gene copies/nucleus) [19].

Similarly, the predictive significance of EGFR gene amplification is also confusing and uncertain. One study of 47 patients with metastatic colorectal cancer treated with a cetuximab-based regimen showed that EGFR gene copy gain, as assessed by fluorescence in situ hybridization, had no correlation with objective response rate, disease control rate, progression-free survival, or overall survival [23]. Conversely, another study of 173 patients with KRas wild-type metastatic colorectal cancer treated with a cetuximab-based regimen found that EGFR amplification/increased EGFR copy number, present in 17.7% of patients, was associated with response to anti-EGFR therapy [24]. These conflicting results may be related to the fact that there are no established guidelines for EGFR gene amplification. But since there are no guidelines, testing for EGFR gene amplification in colorectal cancer is not routinely performed.
In addition to molecular alterations of the EGFR gene, activation of EGFR downstream effectors can lead to tumor formation/progression. Specific alterations can impact prognosis and predict response to anti-EGFR therapy.

### 3.3. KRas Mutations
The KRas proto-oncogene encodes a 21-kDa guanosine 5’-triphosphate- (GTP-) binding protein at the beginning of the MAPK signaling pathway. Somatic KRas mutations are found in many cancers, including 30%–40% of colorectal cancers, and are an early event in carcinogenesis [25–29]. KRas mutations, most commonly codon 12/13 missense mutations, lead to constitutive activation of the KRas protein by abrogating GTPase activity. These mutations result in unregulated downstream signaling that will not be blocked by antibodies that target the EGFR receptor.

The prognostic significance of KRas mutations is controversial. KRas mutation status is associated with shorter survival in some studies [28, 30–32], but not others [29, 32, 33]. The results of one study, which showed increased mortality with codon 13 G-A mutations but not with KRas mutations in general, suggest that prognosis may be related to specific mutations in the KRas gene [28]. Although not predictive of outcome with standard chemotherapy, KRas mutation status is a strong predictive marker of resistance to EGFR-targeted therapy in patients with metastatic colorectal cancer (i.e., KRas mutations predict a lack of response to anti-EGFR monoclonal antibodies cetuximab and panitumumab) [34–39]; this topic is discussed in detail in another paper in this series, “Impact of KRas mutations on management of colorectal cancer” by Sullivan and Kozuch.

### 3.4. BRAF Mutations
The BRAF gene encodes a serine-threonine protein kinase that is downstream of KRas in the MAPK signaling pathway. BRAF mutations occur in 5–22% of all colorectal cancers [40, 41]. When separated by microsatellite instability status, BRAF mutations are present in 40–52% of colorectal cancers that arise through the microsatellite instability pathway (MSI) pathway (microsatellite unstable tumors) [41–44], but only 5% of cancers are microsatellite stable [42]. The most frequently reported BRAF mutation is a valine-to-glutamic acid amino acid (V600E) substitution [45]. BRAF mutations are mutually exclusive with KRas mutations [41].

Unlike KRas mutations, BRAF mutations do have an impact on prognosis and survival. In some studies, the effect is dependent upon the microsatellite status of the colorectal cancer. Patients with a BRAF mutation in a microsatellite-stable colon cancer have significantly poorer survival than those without the mutation, but the BRAF status does not affect survival of patients with microsatellite-unstable tumors [29, 42]. In patients with metastatic KRas wild type tumors, BRAF mutations have been associated with shorter progression-free and shorter overall survival [24]. BRAF status also predicts response to anti-EGFR therapy. Of metastatic colorectal cancers that are found to be KRas wild type at codons 12/13, 5% to 15% can harbor BRAF mutations and show resistance to anti-EGFR therapy [46, 47]. The predictive role of BRAF mutations is further covered in another article in this series, “Impact of KRas mutations on management of colorectal cancer.”

### 3.5. The PI3K Pathway-PIK3CA Mutations and Expression of PTEN and p-AKT
The PI3K-AKT pathway can be deregulated by activating mutations in the PIK3CA gene (p110 subunit), by inactivation (often by epigenetic mechanisms) of the phosphatase and tensin homolog (PTEN) gene, or by activation of AKT [1, 48]. The PIK3CA gene encodes phosphatidylinositol 3-kinase (PI3K), a key signal transducer in the PI3K-AKT pathway. Mutations in PIK3CA occur in 14% to 18% of colon cancers, and most mutations involve hotspots on exons 9 and 20 [47, 49]. Interestingly, there is a strong association between PIK3CA exon 9 mutations and KRas mutations [47]. As a prognostic marker, PIK3CA mutations are associated with shorter cancer-specific survival, but this effect may be limited to patients with KRas wild-type tumors [49]. Briefly, as a predictive marker, only PIK3CA exon 20 mutations appear to be associated with worse outcome after cetuximab [47].

The PTEN gene encodes a protein tyrosine phosphatase enzyme (PTEN) that dephosphorylates phosphatidylinositol-3,4,5 triphosphate (PIP3) and thereby inhibits PI3K function [1]. Loss of PTEN results in constitutive activation of the PI3K-AKT pathway. PTEN mutations and loss of heterozygosity (LOH) of the PTEN locus have been reported in 13%–18% and 17%–19% of colon cancers, respectively [50, 51]. It appears that loss of PTEN has prognostic value. Loss of PTEN protein expression (assessed by IHC) is associated with shorter overall survival in patients with KRas wild-type tumors [24]. It appears that there is an association with PTEN mutations/LOH with MSI status, but the current published results are conflicting [50, 51]. PTEN protein inactivation may also be a negative predictor of response to anti-EGFR therapy [22, 52].

AKT is a major downstream effector of PI3K. A recent study by Baba et al. examined the role of activated (phosphorylated) AKT expression in a large cohort of colorectal cancers [48]. They demonstrated that p-AKT expression is associated with early stage disease and good prognosis. They also showed that p-AKT expression is associated with PIK3CA mutation, as expected from their relationship in the EGFR pathway, but that the prognostic effect of p-AKT expression was independent of PIK3CA mutation. It is possible that p-AKT expression could serve as positive prognostic marker in patients with colorectal cancer.

In summary, the EGFR signaling pathway is a complex and tightly regulated process that is involved in growth, proliferation, and survival of normal cells. When this system goes awry and unchecked, it can lead to growth, proliferation, survival, and metastasis of neoplastic cells. Alterations within the EGFR signaling cascade, such as gene mutations, gene amplification, and protein overexpression, have been shown to contribute to colorectal carcinogenesis. Some alterations also portend a poor prognosis in patients with colorectal cancer. Due to the complex interaction of EGFR and its downstream regulators, the study of individual
components of this pathway often yields conflicting results, as noted in this paper. Hence, there are still many questions that need to be answered before we can fully understand the impact of the EGFR signaling pathway on colorectal carcinogenesis and the prognosis of patients with colorectal cancer.

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MicroRNA Expression in Selected Carcinomas of the Gastrointestinal Tract

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MicroRNAs (miRNAs) comprise a recently discovered class of small, 18–25 nucleotide, noncoding RNA sequences that regulate gene expression at the posttranscriptional level by binding to and inhibiting the translation of target messenger RNAs (mRNAs). Over 1,000 human miRNAs have been identified to date, and many have tissue-specific expression profiles. Several studies have shown that miRNAs demonstrate characteristic patterns of expression in cancers. Some species show overexpression in cancers relative to nonneoplastic tissues, whereas others display decreased expression, similar to oncogenes and tumor suppressor genes, respectively [1–9]. Emerging data indicate that virtually every type of human malignancy displays dysregulated miRNA expression, and, in fact, potential applications of miRNA expression profiling to the diagnosis, prognosis, and treatment of gastrointestinal cancers have been the subject of extensive recent investigation. Some investigators have suggested that panels of miRNAs may be used for diagnostic purposes among patients with suspected gastrointestinal malignancies, and a subset of these represent prognostically important markers or even potential therapeutic targets.

1. Introduction

MicroRNAs are small, 18–25 nucleotide, noncoding RNA sequences that regulate gene expression at the posttranscriptional level by binding to and inhibiting translation of target messenger RNAs (mRNAs). Over 1,000 human miRNAs have been identified to date, and many have tissue-specific expression profiles. Several studies have shown that miRNAs demonstrate characteristic patterns of expression in cancers. Some species show overexpression in cancers relative to nonneoplastic tissues, whereas others display decreased expression, similar to oncogenes and tumor suppressor genes, respectively [1–9]. Emerging data indicate that virtually every type of human malignancy displays dysregulated miRNA expression, and, in fact, potential applications of miRNA expression profiling to the diagnosis, prognosis, and treatment of gastrointestinal cancers have been the subject of extensive recent investigation. Some investigators have suggested that panels of miRNAs may be used for diagnostic purposes among patients with suspected gastrointestinal malignancies, and a subset of these represent prognostically important markers or even potential therapeutic targets.

The purpose of this paper is to provide the readership with a comprehensive overview of published data regarding miRNA expression in gastrointestinal malignancies with emphasis on colorectal, pancreatic, and hepatocellular carcinomas.

2. Overview

MicroRNAs were first recognized as regulatory agents of gene expression in 1993 when they were discovered in Caenorhabditis elegans [10]. Each miRNA molecule can potentially bind to either the 3′- or 5′-untranslated region (UTR) of hundreds of mRNAs by sequence complementarity. Binding of miRNA to mRNA suppresses expression by either inducing mRNA degradation or inhibiting translational machinery. Primary miRNA transcripts within the nucleus are processed by the nuclear RNAse III complex, Drosha, to become miRNA precursors termed “pre-miRNAs”. Pre-miRNAs are exported to the cytoplasm where the endoribonuclease, Dicer, processes them into mature miRNAs. These mature molecules are subsequently integrated into the RNA-induced silencing complex (RISC), the functional unit of which inhibits mRNA translation [11, 12].
A growing body of evidence indicates that a subset of miRNAs are functionally important to the development of human cancers. Many investigators have identified tumor-specific miRNA signatures that accurately distinguish malignancies from benign tissues in multiple different sites, suggesting that some miRNAs are oncogenic, and their potency depends on other gene mutations that are present in the tumor. Manipulation of miRNAs in cancer cell lines directly affects cell proliferation and apoptosis, and many researchers have demonstrated links between miRNA dysregulation and cell signaling pathway abnormalities [9, 13–15]. Thus, miRNAs comprise a recently described class of molecules that contributes to cancer formation through interactions with miRNAs derived from oncogenes and tumor suppressor genes.

3. MicroRNAs in Colorectal Cancer

MicroRNA expression in colon cancer and nonneoplastic colonic tissues has been extensively studied (Table 1). Cummins et al. performed serial analysis of gene expression (miRAGE) in colorectal cancer cell lines and identified 133 miRAGE tags that corresponded to previously unrecognized miRNAs. They also detected differential expression of 52 miRAGE tags in colon cancer cells relative to normal colonic epithelium. These results provided evidence that the number of miRNAs in the human genome is likely much larger than had been previously predicted and that their expression is frequently dysregulated in colorectal cancer [25]. Subsequent studies provided data indicating that miRNA dysregulation is important to colon cancer development. Bandres et al. studied expression of 156 miRNAs in colon cancer cell lines as well as paired tumoral and nontumoral tissues. They identified a subset of 13 differentially expressed species [16]. Wang et al. used miRNA microarrays to identify 12 miRNAs that were upregulated in colon cancer and 2 that were downregulated compared to nonneoplastic colonic tissues [17].

The expression levels of several miRNA species have been associated with clinicopathologic features and prognosis in colon cancer. MicroRNA-31 was first identified by Bandres et al. as one of the most substantially dysregulated miRNAs in colon cancer cell lines and resected colon cancers. The authors of that study found that miR-31 expression was significantly higher in stage IV tumors compared to stage II carcinomas [16]. Wang et al. later demonstrated an association between miR-31 upregulation and advanced TNM stage as well as deeper invasion of the primary tumor [26]. Slaby et al. failed to identify any correlation between miR-31 expression and tumor stage in their analysis of 29 colon carcinomas, but they did note that miR-31 levels were significantly higher in high-grade carcinomas, compared to low-grade tumors [19]. MicroRNA-21, a species with antiapoptotic properties, is dysregulated in many human cancers including tumors of the head and neck, lung, breast, prostate, brain, thyroid, pancreas, stomach, colon, and esophagus [9, 27–32]. Its high expression has been associated with regional lymph node and distant metastases in colorectal cancer patients [19]. Schetter et al. analyzed 197 colonic adenocarcinomas using microarray assays and qRT-PCR and found that high miR-21 levels predicted poor survival prognosis and higher TNM stage [21]. This same group later demonstrated a relationship between high miR-21 expression and increased levels of IL-6, a proinflammatory cytokine and lower levels of IL-12a in colonic adenocarcinomas. They postulated that IL-6 drives miR-21 expression whereas IL-12a is a negatively regulated target of miR-21 [21]. Presumably, IL-12a activity is important for host resistance to malignancy. Thus, its downregulation by miR-21 may account for some of the negative impact of miR-21 on prognosis among patients with colorectal cancer. Finally, miR-145 is normally expressed in colonic epithelium, but it shows decreased expression in colon cancer [23]. Decreased miR-145 is more commonly observed in tumors of the proximal colon and those of large size (>50 mm) [22]. Akao et al. found decreased expression of miR-143 and miR-145 in adenomas and carcinomas, but they did not find any correlation between their expression and any other clinical prognostic factors, indicating that these miRNAs may primarily contribute to initiation, but not progression, of colonic tumorigenesis. Notably, synthetic miR-143 has a suppressive effect on growth of xenografted tumors comprised of human colon cancer cells [33].

Expression of a number of other miRNA species has been reported to correlate with clinicopathologic features and prognosis among patients with colon cancer. Schepele et al. found that stage II colon cancers with high miR-320 or miR-498 expression showed significant differences with respect to progression-free survival compared to tumors with low expression of these species [18]. High expression of miR-200c has also been reported to predict shorter survival and is associated with frequent p53 mutations [20]. Diaz et al. studied 110 patients with colon cancer and reported that downregulation of miR-106a predicted shorter disease-free survival [34]. Sarver et al. identified 6 miRNAs (HS-29, miR-135b, miR-32, miR-33, miR-542-5p, and miR-96) that were more highly expressed in stage IV microsatellite stable cancers relative to stage II tumors [22]. Huang et al. studied nonneoplastic colonic mucosa adjacent to colon cancers in three patients with lymph node metastases and three patients with node negative disease. They found 6-fold higher expression of miR-137 in lymph node positive tumors compared to node-negative cases [35]. Finally, Yantiss et al. studied miRNA expression in colorectal cancers obtained from 24 patients <40 years of age and 45 patients >40 years old. They found significantly increased expression of miR-21, miR-20a, miR-145, miR-181b, and miR-203 in tumors from young patients compared to older adults [36].

Data from several studies have documented interactions between specific miRNA species, oncogenes, and tumor suppressor genes relevant to colonic carcinogenesis. Chen et al. observed an inverse correlation between miR-143 and KRAS expression in 13 colonic adenocarcinomas. They also used semiquantitative RT-PCR to show that KRAS transcript levels were decreased in cell lines transfected with pre-miR-143 whereas addition of anti-miR-143 oligonucleotides increased KRAS transcript levels. These findings suggest that miR-143 downregulation in colon carcinoma promotes cancer cell growth by disinhibiting KRAS translation [37].
MicroRNAs also impact the Wnt signaling pathway. The adenomatous polyposis coli (APC) gene normally functions as a tumor suppressor by regulating Wnt signaling. In the absence of functional APC, β-catenin accumulates in the cytoplasm and is transported to the nucleus, where it facilitates transcription of genes involved in proliferation, such as cyclin D1. Germline APC mutations are responsible for familial adenomatous polyposis syndrome, and biallelic inactivation of APC occurs in the majority of sporadic colorectal adenocarcinomas [38–41]. Transduction of colon cancer cell lines with miR-135a and miR-135b results in diminished APC expression and accumulation of β-catenin. Colon cancers with high levels of miR-135a and miR-135b show lower APC expression. In this situation, qRT-PCR data demonstrate reduced APC mRNA, suggesting that miR-135a and miR-135b regulate the Wnt signaling pathway by promoting mRNA decay [42].

MicroRNA-34, a species that is lost in multiple types of cancer including those of the colon and pancreas, is inductive by p53, and its overexpression is associated with p53 effects including cell cycle arrest and apoptosis [43]. Guo et al. found that restoration of miR-126, a species that is lost in multiple types of cancer, impedes cancer cell growth by targeting the p85B subunit of phosphatidylinositol-3-kinase (PI3K). Phosphatidylinositol-3-kinase activates AKT, a protein kinase involved in the PI3K/AKT/mTOR pathway, which triggers a variety of downstream responses related to cell growth, proliferation, and motility. Presumably, loss of miR-126 removes a critical checkpoint in the PI3K/AKT/mTOR pathway and facilitates tumor growth [24]. Continued research will likely uncover additional regulatory roles for miRNAs in colorectal carcinogenesis and other neoplasms throughout the gastrointestinal tract.

Most colorectal cancers are characterized by aneuploidy, allelic imbalance, and mutations in KRAS, TP53, and APC although approximately 15% of sporadic colon adenocarcinomas develop via microsatellite instability (MSI) and have defective DNA mismatch repair mechanisms. Such tumors are often located in the proximal colon, show high-grade histology, and contain infiltrating lymphocytes. They are generally diploid and have a better prognosis than non-MSI tumors, but they are probably less responsive to conventional 5-flourouracil-based chemotherapy [44, 45]. Patients with germline mutations in one of the mismatch repair genes (MLH1, MSH2, MSH6, and PMS2) are at risk for acquired mutations of the second allele and development of heritable microsatellite unstable carcinomas of the gastrointestinal, genitourinary, and gynecologic tracts, termed Lynch syndrome. Sporadic colorectal carcinomas with MSI may also occur, but in this situation, tumorigenesis results from biallelic epigenetic methylation and silencing of MLH1. Several authors have studied differences in miRNA expression patterns between microsatellite stable (MSS) colon cancers and those with MSI-H (Table 2). Lanza et al. used microarray data to profile 23 MSS and 16 sporadic MSI-H colon cancers. They identified 72 mRNAs and 14 miRNAs that were differentially expressed between these two tumor types and used their combined expression to distinguish between MSS and MSI-H cancers [14]. Sarver et al. studied expression of 735 miRNAs in 12 MSI-H and 68 MSS colon tumors and found that miR-552, miR-592,

**Table 1: Summary of microRNA expression in colorectal cancer.**

<table>
<thead>
<tr>
<th>Study</th>
<th>Differentially expressed miRNAs (no./total)</th>
<th>Most significantly overexpressed miRNAs in colorectal cancer</th>
<th>Most significantly underexpressed miRNAs in colorectal cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bandres et al. [16]</td>
<td>13/156</td>
<td>miR-31, miR-96, miR-133b, miR-135b, miR-145, miR-183</td>
<td></td>
</tr>
<tr>
<td>Wang et al. [17]</td>
<td>14/723</td>
<td>miR-106b, miR-135b, miR-18a, miR-18b, miR-196b, miR-19a, miR-224, miR-335, miR-424, miR-20a*, miR-301b, miR-734a</td>
<td>miR-378, miR-378*</td>
</tr>
<tr>
<td>Schepeler et al. [18]</td>
<td>60/315</td>
<td>miR-20a, miR-310, miR-92, miR-513</td>
<td>miR-145, miR-455, miR-484, miR-101</td>
</tr>
<tr>
<td>Slaby et al. [19]</td>
<td>4/4</td>
<td>miR-31, miR-21</td>
<td>miR-145, miR-143</td>
</tr>
<tr>
<td>Xi et al. [20]</td>
<td>4/10</td>
<td>miR-15b, miR-181b, miR-191, miR-200c</td>
<td>miR-145, miR-143</td>
</tr>
<tr>
<td>Schetter et al. [21]</td>
<td>37/389</td>
<td>miR-20a, miR-21, miR-106a, miR-181b, miR-203</td>
<td>miR-145, miR-143</td>
</tr>
<tr>
<td>Sarver et al. [22]</td>
<td>39/735</td>
<td>miR-135b, miR-96, miR-182, miR-182*, miR-183</td>
<td>miR-135b, miR-96, miR-182, miR-182*, miR-183</td>
</tr>
<tr>
<td>Michael et al. [23]</td>
<td>2/28</td>
<td>miR-93, miR-92, miR-520h, miR-508, miR-505, miR-449, miR-429, miR-384, miR-373, miR-34c, miR-326, miR-25, miR-224, miR-210, miR-200a, miR-19b, miR-19a, miR-18a, miR-183, miR-182, miR-181b, miR-181a, miR-181c, miR-17-5p, miR-148a, miR-141, miR-130b, miR-128a, miR-106b, miR-106a, let-7d</td>
<td>miR-143, miR-145</td>
</tr>
<tr>
<td>Guo et al. [24]</td>
<td>45/262</td>
<td>miR-96, miR-485-5p, miR-422b, miR-342, miR-214, miR-199a, miR-195, miR-150, miR-145, miR-143, miR-133a, miR-126, miR-125b, miR-100</td>
<td></td>
</tr>
</tbody>
</table>

*MicroRNAs in colorectal cancer*
miR-181c, and miR-196b were significantly increased in MSS tumors relative to MSI-H tumors, whereas miR-625 and miR-31 levels were higher in MSI-H tumors [22]. Schepeler et al. compared miRNA expression in 37 MSS relative to 12 MSI-H cancers and identified a 4-miRNA signature (miR-142-3p, miR-212, miR-151, and miR-144) that predicted MSI with 81% specificity and 92% sensitivity [18]. These data indicate that the type of genetic instability in colorectal cancer is reflected at the miRNA level.

5-Flourouracil (5-FU) has been a mainstay of colorectal cancer therapy for the past several decades [46]. Some patients have a suboptimal response to therapy for unclear reasons, so identification of molecular markers that predict the likelihood of a therapeutic response is important. In vitro studies evaluating miRNA expression in colon cancer cell lines treated with 5-FU have generated promising data regarding the potential use of miRNAs as markers of chemosensitivity. Borralho et al. showed that stable expression of miR-143, a species known to be downregulated in colon cancer, was associated with increased death in cell lines after exposure to 5-FU [23, 47]. Others have applied this concept to colon cancer resection specimens. Nakajima et al. used qRT-PCR to study miRNA expression in residual or recurrent colon cancers from 46 patients who were treated with oral 5-FU alone or in combination with cisplatin. Twenty-seven patients who experienced complete disease remission showed a partial response or maintained stable disease after treatment had significantly lower levels of let-7g and miR-181b, compared to 19 patients who suffered disease progression [48]. Schetter et al. analyzed associations between miR-21 expression and therapeutic outcomes in 56 stage II or stage III colorectal cancer patients treated with 5-FU. High miR-21 expression was associated with worse overall survival, lending preliminary support to the notion that miR-21 overexpression predicts a poor response to therapy [21]. Boni et al. investigated associations between polymorphisms in miRNA-containing genomic regions and genes related to miRNA biogenesis and clinical outcome in patients with metastatic colon cancer who were treated with 5-FU and irinotecan. Single-nucleotide polymorphisms in the miR-26-a-1 gene and 5’UTR of pre-miR-100 correlated with better overall survival and disease control, respectively, and both were associated with a prolonged interval to progression [49]. The mechanisms by which miRNAs modulate efficacy of therapy are not understood, but these early data support the hypothesis that changes in miRNA expression levels and in the miRNA genome impact tumor response to therapy.

4. MicroRNAs in Pancreatic Neoplasia

Aberrant miRNA expression has been described in pancreatic ductal adenocarcinoma and benign pancreatic diseases (Table 3). Early studies exploited differences in miRNA expression patterns to distinguish between benign and malignant pancreatic diseases. Bloomston et al. examined 65 pancreatic ductal adenocarcinomas and benign adjacent pancreatic tissue, as well as 42 cases of chronic pancreatitis for miRNA expression. They found 21 miRNAs to be dysregulated in cancer compared to benign tissues and noted that a panel of 11 miRNAs (miR-148a, miR-148b, miR-155, miR-181a, miR-181b, miR-181b-1, miR-181c, miR-181d, miR-21, miR-221, and miR-375) distinguished pancreatic ductal adenocarcinoma from chronic pancreatitis and normal pancreatic tissue [49]. Lee et al. analyzed 28 pancreatic ductal adenocarcinomas and 21 nonneoplastic pancreatic tissues and found miR-155, miR-181b, miR-181c, miR-21, miR-221, and miR-222 to be among the top 20 of 100 miRNAs overexpressed in pancreatic cancer [31]. Szafrańska et al. identified 26 dysregulated miRNAs in pancreatic adenocarcinoma using resection specimens and pancreatic cancer cell lines. They reported that miR-196a upregulation combined with miR-217 downregulation reliably distinguished pancreatic cancer from benign pancreas and chronic pancreatitis [50]. In a later study, this signature correctly identified malignancy in 9/10 fine needle aspiration biopsies of pancreatic cancer [51].

The roles of dysregulated miRNAs in neoplastic pancreatic lesions have also been examined. du Rieu et al. used qRT-PCR to study miRNA expression in pancreatic intraepithelial neoplasia (PanIN) samples from human and mouse pancreas and found that levels of miR-21, miR-221, miR-22, and let-7a increased in the progression of PanIN to carcinoma [52]. Dilhoff et al. found that strong miR-21 expression by in situ hybridization was associated with decreased survival among patients with lymph node-negative pancreatic carcinoma [53]. Ikenaga et al. used qRT-PCR to evaluate miR-203 levels in resection specimens from patients with pancreatic ductal adenocarcinoma (n = 113), chronic pancreatitis (n = 20), and samples of nondiseased pancreas (n = 8) and found higher expression of miR-203 in pancreatic cancer.
Table 3: Summary of microRNA expression in pancreatic ductal adenocarcinoma.

<table>
<thead>
<tr>
<th>Study</th>
<th>Differentially expressed miRNAs (no./total)</th>
<th>Most significantly overexpressed miRNAs compared with normal controls and/or chronic pancreatitis</th>
<th>Most significantly underexpressed miRNAs compared with normal controls and/or chronic pancreatitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bloomston et al. [30]</td>
<td>46/326</td>
<td>miR-221, miR-181a, miR-155, miR-210, miR-213, miR-181b, miR-222, miR-181b-2, miR-21, miR-181b-1, miR-181c, miR-220, miR-181d, miR-223, miR-100-1/2, miR-125a, miR-143, miR-10a, miR-146, miR-99, miR-100, miR-199a-1, miR-10b, miR-199a-2, miR-107, miR-103-2, miR-125b-1, miR-205, miR-23b, miR-23a, miR-96, miR-34, miR-497, miR-203, miR-453, miR-92, miR-93, miR-21</td>
<td>miR-148a, miR-148b, miR-375, miR-494, miR-483, miR-339, miR-218-2, miR-409-3p</td>
</tr>
<tr>
<td>Lee et al. [31]</td>
<td>100/222</td>
<td>miR-221, miR-424, miR-301, miR-100, miR-376a, miR-125b-1, miR-21, miR-16-1, miR-181a, miR-181c, miR-92-1, miR-15b, miR-155, let-7f-1, miR-212, miR-107, miR-24-1, miR-24-2, let-7d</td>
<td>miR-345, miR-142-p, miR-139</td>
</tr>
<tr>
<td>Szafranska et al. [50]</td>
<td>26/377</td>
<td>miR-205, miR-143, miR-145, miR-146a, miR-148a, miR-196b, miR-93, miR-31, miR-210, miR-196a, miR-18a, miR-203, miR-150, miR-155, miR-221, miR-222, miR-223, miR-224</td>
<td>miR-29c, miR-216, miR-217, miR-375, miR-148a, miR-96, miR-148b, miR-141, miR-130b</td>
</tr>
<tr>
<td>du Rieu et al. [52]</td>
<td>7/7</td>
<td>miR-21, miR-221, miR-222, miR-200, miR-205, miR-29c</td>
<td>let-7a</td>
</tr>
</tbody>
</table>

Results of a multivariate analysis showed miR-203 expression to be an independent predictor of poor prognosis in patients who had undergone complete tumor resection [54]. Wang et al. investigated expression of miR-21, -210, -155, and 196a by qRT-PCR in the sera of 49 patients with pancreatic adenocarcinoma and 36 healthy controls and found higher expression of all four markers in sera of pancreatic cancer patients [55]. In a later study, Kong et al. analyzed serum levels of miR-196a, miR-21, and miR-155 in 35 patients with pancreatic adenocarcinoma, 15 patients with chronic pancreatitis, and 15 healthy controls. They found that higher miR-21 expression distinguished cancer patients from those with chronic pancreatitis and healthy subjects, whereas miR-155 and miR-196a discriminated between patients with chronic pancreatitis and healthy controls. They also noted that serum miR-196a levels were significantly higher in patients with unresectable cancer than in those amenable to surgery and that higher miR-196a levels predicted shorter survival in pancreatic cancer patients [56]. Others have shown increased miR-196a expression to be associated with a 2-year survival of 17%, compared to 64% among tumors with low expression of this marker [30].

The relative tissue-specificity of miRNAs makes them attractive targets for molecular therapy among patients with pancreatic cancer. Park et al. analyzed the effects of miR-21 and miR-221 antisense oligonucleotides on pancreatic cancer cell lines and found that treated cells showed increased apoptosis and cell cycle arrest compared to cells treated with control oligonucleotides. MicroRNA-21 targets two tumor suppressor molecules, PTEN and RECK, both of which were found to be increased in extracts from cell lines treated with antisense oligonucleotides to miR-21. Similarly, p27, the target of miR-221, increased when this molecule was inhibited. Park et al. also found that cells pretreated with antisense sequences against miR-21 and miR-221 showed decreased viability by colorimetric analysis following gemcitabine treatment compared to those treated with control oligonucleotides [57].

Few studies have investigated miRNA expression in pancreatic endocrine and acinar tumors. Roldo et al. performed microarray and northern blot analyses of 40 endocrine tumors, 4 acinar cell carcinomas, and 12 samples of nonneoplastic pancreas. They found that stable expression of miR-103 and miR-107, in combination with a lack of miR-155 expression, discriminated all tumor samples from nonneoplastic tissues. They also identified 10 miRNAs that distinguished endocrine from acinar tumors and 28 miRNA species that were aberrantly increased in both tumor types [29]. These preliminary data suggest that altered miRNA expression occurs in endocrine and acinar neoplasms of the pancreas.

5. MicroRNAs in Liver Disease

Early studies evaluating miRNA expression in hepatocellular carcinoma identified approximately 69 dysregulated species...
(Table 4) [58–63]. Many of those miRNAs, including miR-122, miR-221, and miR-222, were later recognized as carcinogenic catalysts and prognostic markers in hepatocellular carcinoma. MicroRNA-122 is the most abundant miRNA in hepatic parenchyma and is relatively specific for hepatocyte differentiation, showing rare expression outside of liver [64–66]. Downregulation of miR-122 is frequently observed in hepatocellular carcinoma, and hepatocellular carcinoma cell lines treated with miR-122 oligonucleotides show increased apoptosis and decreased viability [58, 59, 67–72]. Coulouarn lines treated with miR-122 oligonucleotides show increased proapoptotic protein, Bmf, in hepatocellular carcinoma cell lines transfected with anti-miR-221 compared to controls. Conversely, cell lines treated with miR-221 showed decreased CDKN1C/p57 and CDKN1B/p27 protein levels [77]. Finally, Mneg et al. reported that inhibition of miR-21 in hepatocellular carcinoma cell lines increased expression of PTEN and decreased tumor cell proliferation, suggesting that increased miR-21 levels promote carcinogenesis [78]. Other identified, but less-well-studied, modulators of apoptosis in hepatocellular carcinoma include miR-29, miR-15b, miR-152, miR-101, and the miR-106b-25 cluster [75, 79–82]. Tsai et al. reported lower levels of miR-122 in hepatocellular carcinomas with intrahepatic metastases compared to solitary tumors [74].

The roles of microRNA species in hepatocellular carcinogenesis and their molecular targets are under current investigation. Young et al. found that high miR-222 levels correlated with advanced tumor stage and shorter overall survival in patients with hepatocellular carcinoma independent of stage. They also noted PI3K/AKT/mTOR pathway inhibition occurred in hepatocellular carcinoma cell lines transfected with anti-miR-222 [62]. MicroRNA-221, another frequently dysregulated species in hepatocellular carcinoma, participates in the modulation of key molecules related to hepatocarcinogenesis. Pianu et al. found that expression levels of the cyclin-dependent kinase inhibitor, p27, and the PI3K/AKT/mTOR pathway regulator, DDIT4, were decreased in liver cancer cell lines that overexpressed miR-221 [61]. Gramantieri et al. showed an inverse correlation between miR-221 upregulation and levels of the proapoptotic protein, Bmf, in hepatocellular carcinoma samples [76]. Fornari et al. showed increased CDKN1C/p57 and CDKN1B/p27 protein levels by western blot analysis in hepatocellular carcinoma cell lines transfected with anti-miR-221 compared to controls. Conversely, cell lines treated with miR-221 showed decreased CDKN1C/p57 and CDKN1B/p27 protein levels [77]. Finally, Mneg et al. reported that inhibition of miR-21 in hepatocellular carcinoma cell lines increased expression of PTEN and decreased tumor cell proliferation, suggesting that increased miR-21 levels promote carcinogenesis [78]. Other identified, but less-well-studied, modulators of apoptosis in hepatocellular carcinoma include miR-29, miR-15b, miR-152, miR-101, and the miR-106b-25 cluster [75, 79–82].

Ji et al. evaluated a cohort of 214 patients with hepatocellular carcinoma and found that tumors with reduced miR-26 expression had a favorable response to adjuvant therapy with interferon alpha, whereas those with high miR-26 did not respond to therapy, suggesting that miR-26 may be used to select patients who may benefit from interferon alpha treatment [83]. Connelly et al. reported that miR-21 and the miR-17-92 polycistron are consistently upregulated in human and animal hepatocellular carcinoma cell lines and that their inhibition by antisense oligonucleotides causes reduced tumor cell proliferation [84].

Recent studies have established a role for miRNAs in regulation of hepatitis C virus (HCV) infection and offer promise for new treatment modalities. The most frequently implicated miRNA in HCV modulation is the liver-specific species, miR-122. Jopling et al. first described a physical interaction between miR-122 and the HCV genome by showing that miR-122 binds to the 5′ UTR of viral RNA and stimulates viral replication [85]. Henke et al. showed that miR-122 drives HCV translation by enhancing the association between a small ribosomal subunit and HCV RNA [86]. Both mechanisms of HCV potentiation were later validated by Jangra et al. who demonstrated that viruses with mutations in miR-122 binding sites failed to replicate [87]. Young et al. reported decreased viral replication in liver cells.

### Table 4: Summary of microRNA expression in hepatocellular carcinoma.

<table>
<thead>
<tr>
<th>Study</th>
<th>Differentially expressed miRNAs (no./total)</th>
<th>Most significantly overexpressed miRNAs compared with nonneoplastic liver (no disease or chronic viral hepatitis)</th>
<th>Most significantly underexpressed miRNAs compared with nonneoplastic liver (no disease or chronic viral hepatitis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murakami et al. [58]</td>
<td>7/180</td>
<td>miR-18, miR-224</td>
<td>miR-199a∗, miR-195, miR-199, miR-200a, miR-125a</td>
</tr>
<tr>
<td>Li et al. [59]</td>
<td>84/509</td>
<td>miR-106b, miR-15b, miR-18a, miR-221, miR-222, miR-224</td>
<td>miR-125b, miR-101</td>
</tr>
<tr>
<td>Ladeiro et al. [67]</td>
<td>130/250</td>
<td>miR-224, miR-200c, miR-203, miR-21, miR-222, miR-10b</td>
<td>miR-422b, miR-122a</td>
</tr>
<tr>
<td>Varnholt et al. [60]</td>
<td>29/80</td>
<td>miR-122, miR-100, miR-10a</td>
<td>miR-198, miR-145</td>
</tr>
<tr>
<td>Li et al. [75]</td>
<td>8/8</td>
<td>miR-17-5p, miR-18a, miR-19a, miR-20a, miR-92-1, miR-106b, miR-93, miR-25</td>
<td></td>
</tr>
<tr>
<td>Pineau et al. [61]</td>
<td>12/215</td>
<td>miR-106b, miR-21, miR-210, miR-221, miR-222, miR-34a, miR-425, miR-519a, miR-93, miR-96</td>
<td>let-7c</td>
</tr>
</tbody>
</table>
treated with inhibitors of miR-122 and suggested that these small molecules may represent a new target for HCV therapy, which has already been successfully tested in HCV-infected chimpanzees [72, 88].

The potential impact of miRNA analysis on patient selection for specific therapies was underscored by Sarasin-Filipowicz et al. These authors assessed miR-122 levels by qRT-PCR in pre- and posttreatment liver biopsies from 42 patients with HCV. They found that patients with decreased miR-122 levels in pretreatment liver biopsies showed a poor response to interferon therapy [89]. Other miRNA species such as miR-24, miR-149, miR-638, and miR-1181 have also been implicated in HCV-related liver disease and may facilitate viral entry, replication, and propagation [90].

MicroRNA dysregulation also occurs in association with hepatitis B virus (HBV) infection and may provide clues to the pathogenesis of HBV-related disease in infected patients. Yang et al. found that miR-602 expression increased with progression of HBV-related hepatitis to cirrhosis and hepatocellular carcinoma and noted that the tumor suppressor gene RASSF1A was inhibited in cell lines that highly expressed miR-602 [91]. Ura et al. studied 12 patients with HBV-related hepatocellular carcinoma and 14 with HCV-related hepatocellular carcinoma. They identified 19 differentially expressed miRNAs between patients with HBV and HCV infection. Microarray analysis also identified separate target genes for HBV- and HCV-related cancers. MicroRNAs important to HBV-related carcinoma regulate genes involved in cell death, DNA damage, recombination, and signal transduction whereas those important to HCV-related carcinoma were related to immune response, antigen presentation, cell cycle, and proteasome and lipid metabolism [92]. These findings provide insight into the differences between HBV- and HCV-infection and disease progression and may help identify potential therapeutic target molecules in the future.

6. MicroRNAs and In Vitro Cancer Models

Several strategies utilizing miRNAs as in vivo therapeutic targets are currently under development. Use of antisense oligonucleotides has been most extensively studied in vitro, and was recently shown to be an effective suppressor of miRNA expression in vivo. Krutzfeldt et al. engineered synthetic RNA analogues, termed “antagomiRs,” to miR-16, miR-122, miR-192, and miR-194. These compounds were administered to mice intravenously and corresponding miRNA levels were measured by northern blot assay 24 hours after injection. These authors reported a marked reduction in target miRNA levels in various tissues including liver, lung, kidney, heart, intestine, fat, skin, bone marrow, muscle, ovaries, and adrenal glands [93]. Locked nucleic acid (LNA) constructs represent another promising approach to suppressing miRNA expression. These molecules are nucleic acid analogues that are “locked” by a methylene bridge connecting the 2’O and 4’C atoms. This structural modification enables LNA oligonucleotides to bind complementary nucleotide sequences with high affinity and excellent mismatch discrimination. Elmen et al. administered an LNA-antimiR to African green monkeys in order to study its effect on plasma cholesterol levels and miR-122 levels in liver tissue by northern blot analysis. They observed a dose-dependent decrease in total plasma cholesterol and depletion of mature miR-122 in liver biopsies from these monkeys [94]. Finally, some investigators have employed adenovirus vectors to increase expression of tumor-suppressor miRNAs. Kota et al. showed that the adenovirus vector-mediated introduction of miR-26, a species downregulated in hepatocellular carcinoma, into mice with liver cancer caused cancer cell apoptosis and tumor regression. This therapy had no adverse effect on benign hepatocytes, underscoring the potential applications of this approach [95]. Future in vivo progress in this field will depend upon increased understanding of miRNA function in mammals, improved chemical design of antimiRs and synthetic miRNAs, and development of more efficient methods for delivery of these molecules to target tissues.

7. Conclusion

MicroRNAs represent an important class of molecules with profound diagnostic and therapeutic implications. Emerging evidence suggests that they may be useful diagnostic adjuncts that aid identification of tumors of unknown origin or even ascertain the presence of malignancy in scant biopsy specimens or sera of patients with suspected cancer. Specific miRNA expression profiles clearly correlate with prognosis, so it is highly likely that miRNA analysis will play an important role in determining the management of patients in the future. Preliminary studies utilizing antisense oligonucleotides against cancer-specific miRNAs have shown that some tumors respond to therapy while minimally damaging healthy tissues. These findings suggest that targeted therapies against selected miRNAs represent a new treatment modality for patients with gastrointestinal malignancies. Advances in this field have improved our understanding of the heterogeneity of human malignancies and will contribute to the growing trend toward individualized management strategies for cancer patients.

References


Review Article

Hereditary Diffuse Gastric Cancer: Multidisciplinary Case Report with Review of the Literature

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Hereditary diffuse gastric cancer (HDGC) is a rare, inherited cancer syndrome with at least one fourth of HDGC patients having an autosomal dominantly inherited mutation of \( CDH1 \) (E-Cadherin). Penetrance is relatively high (70–80% lifetime risk for gastric cancer). It is important for pathologists to recognize the syndrome’s phenotype in early gastric lesions: patchy intramucosal signet ring cells often associated with pagetoid spread. Due to the insidious nature of this lesion, surveillance is limited and currently prophylactic gastrectomy is an option chosen by many HDGC patients. We present a case report from a multidisciplinary team of authors with a review of the literature that includes the updated guidelines for \( CDH1 \) genetic testing.

1. The Case

An asymptomatic 36-year-old male presented for an elective, increased risk, upper endoscopy (EGD). The patient’s remarkable history included at least five family members diagnosed with gastric carcinoma, four known to have occurred at an early age (Figure 1). He had a documented mutation in the \( CDH1 \) (E-Cadherin) gene (1212delC) identified approximately one month prior to this surveillance EGD. The EGD was essentially normal. The stomach was insufflated and decompressed without difficulty and there was no visual evidence of mucosal changes. EGD biopsies were read as gastric body mucosa without diagnostic abnormality and were negative for \( Helicobacter pylori \), intestinal metaplasia, dysplasia, and malignancy.

Three months later, following genetic counseling, the patient was admitted for an exploratory laparotomy with a prophylactic total gastrectomy with D2 lymphadenectomy and end-to-side Roux-en-Y esophagojejunostomy and needle catheter feeding jejunostomy. The patient’s sister and two cousins had recently undergone similar prophylactic procedures.

Surgical exploration showed no evidence of metastatic disease. Gross pathological examination of the stomach revealed intact, pink mucosa with normal rugae and no masses. The entire surgical margins, multiple (>40) gastric sections from all anatomic zones, and the entire perigastric adipose tissue were submitted for histologic review. Histologic examination revealed multifocal, intramucosal signet ring cell adenocarcinoma with focal pagetoid spread of the signet ring cells in preserved fundic glands (Figure 2(a)). The sections were negative for \( Helicobacter pylori \) and \( Helicobacter pylori \). All margins and lymph nodes were negative for tumor (AJCC pathologic stage pT1a pN0).

The patient had an uneventful postoperative recovery with an upper gastrointestinal study done on postoperative day #5 that showed no anastomotic leaks or strictures. He was discharged on postoperative day #7. Followup examinations revealed extensive weight loss (approximately 50 lbs) but the patient was otherwise doing well.
2. Molecular Carcinogenesis

Only a small number, 2–8%, of all gastric carcinomas arise from inherited gastric cancer syndromes [1, 2]. The majority of families with autosomal dominant familial gastric carcinoma will have the diffuse, poorly differentiated (linitis plastica) morphologic subtype and are referred to as hereditary diffuse gastric cancer (HDGC). A germline mutation in the tumor suppressor gene CDH1 (E-Cadherin) is identified in approximately 25–48% of individuals with HDGC [3, 4]. E-Cadherin is an epithelial cell-cell adhesion molecule essential to cell differentiation and normal epithelial cell architecture. It is therefore not surprising that germline mutations in E-Cadherin are highly specific for families who meet HDGC criteria and has not been described in families with inherited intestinal type/morphology gastric carcinoma [3, 5, 6].

In 1999, the International Gastric Cancer Linkage Consortium (IGCLC) met to first define criteria required to select those patients appropriate to receive mutational screening for HDGC [5]. Strict application of these 1999 IGCLC criteria found approximately one third of screened patients to be heterozygous for a germline, point or frameshift, mutation of the CDH1 gene on chromosome 16q22.1 [6, 7]. This implies that there are either currently unidentifiable CDH1 mutations or other genes causing HDGC in some families. Medical management recommendations are particularly difficult in these families with undetectable CDH1 mutations. There are at least twenty-seven documented inactivating (truncating) CDH1 mutations scattered at various exons along the E-Cadherin gene described in a diverse ethnic population [3, 8–10]. In a recent study addressing patients that met HDGC criteria but lacked CDH1 germline point mutations, Oliveira et al. found that 6.5% of their study patients had large deletions affecting the CDH1 locus by using multiplex ligation-dependent probe amplification (MLPA) [7]. Therefore, analysis for large...
genomic deletions using alternative techniques such as MLPA or array comparative genomic hybridization (CGH) should be explored in highly suspicious cases in which standard DNA sequencing is negative for point mutations.

The current hypothesis for how HDGC susceptibility cancers lose their CDH1 heterozygosity and thus their E-Cadherin expression follows the “two-hit” mutation theory [11]. Hypermethylation of the CDH1 promoter is the most common cause of inactivation of the second allele; however, mutation and loss of heterozygosity (LOH) are also well-documented culprits [11–13]. Interestingly, the mechanisms of the “2nd hit” may differ in primary tumors versus metastases, an important consideration for therapeutics. The morphologic expression of the second hit is multifocal clusters of tumor cells that have lost or show abnormal (reduced) E-cadherin expression (Figure 2(b)). The subsequent step, progression to invasion of the submucosa, is less well defined. The current line of thought involves an integrated role between additional genetic events and changes in microenvironment. Unfortunately, there are no current means of predicting the time course between tumor expansion and submucosal invasion. The CDH1 mutation carries a high penetrance with carriers of germline mutations having an estimated lifetime risk of 67% in men and 83% in women to develop gastric carcinoma [4, 14]. In HDGC families, the most commonly described malignancy other than diffuse gastric carcinoma is lobular breast adenocarcinoma. Female carriers of a germline CDH1 mutation have a 40% lifetime risk of developing this subset of breast cancer [14]. In general, susceptibility cancers in HDGC occur at a relatively early age, with the average age of presentation of diffuse gastric carcinoma being 38 years [15].

### 3. Clinicopathologic Management

The IGCLC guidelines were first developed in 1999 to support clinical management of families felt to be predisposed to gastric carcinoma [5]. This multidisciplinary group has recently provided us with an updated, somewhat broadened version of their original guidelines as to which patients should be offered molecular genetic testing (Table 1) [4]. Any patient who meets the minimum requirements for HDGC listed in Table 1 should be offered genetic counseling and testing for the CDH1 mutation. Health care professionals experienced in cancer genetics must provide those patients that choose to undergo this testing pre- and posttesting genetic counseling.

In general, annual endoscopy using a white light high-definition endoscope is recommended for surveillance of HDGC patients. Using histopathology mapping in six gastrectomy specimens from New Zealand Maori HDGC families, Charlton et al. showed a preferential pattern of intramucosal diffuse gastric carcinoma in the body-antral transitional zone [16]. Based on their study, they proposed a targeted approach to endoscopy with the goal of minimizing sampling error. However, results from prophylactic gastrectomy specimens from other areas of the world did not show similar findings [17–21]. Due to the discrepancy in study results regarding localization of early gastric lesions, multiple gastric biopsies representing all of the major gastric anatomic zones is recommended. The HDGC endoscopic protocol provided by the most recent consensus guidelines suggests the following biopsies: a single antral biopsy taken first for surveillance of *H. pylori* status, any focal lesions, and in addition at least three biopsies from each anatomical area (prepyloric area, gastric antrum, transitional zone, gastric body, gastric fundus, and gastric cardia) [4]. This extensive sampling is driven by the insidious phenotype of this disease (patchy, diffuse growth pattern of gastric carcinoma under endoscopically normal gastric mucosa), which leads to low sensitivity in currently available surveillance procedures.

Our HDGC patient was found to have occult intramucosal signet ring cell adenocarcinoma on resection despite having a normal endoscopic appearance to the stomach and no diagnostic findings by random endoscopic biopsies. Upon review of the literature representing different, independent HDGC families, the identification of indolent gastric cancer is consistent with the pathology found in the majority of cases following prophylactic gastrectomy [19, 20, 22]. In light of the inherent limitations to the current screening procedures/tests available for HDGC patients, a prophylactic gastrectomy is not only a reasonable option but some may argue a life saving one. The estimated 30-day postgastrectomy mortality rate is cited as 3–6% [23] and most likely reaches even smaller numbers when a gastrointestinal surgeon who routinely performs gastrectomies and other major surgeries performs the procedure. To put this in perspective, the 5-year mortality rate in patients with symptomatic, invasive gastric carcinoma is 90% [24].

However, this procedure is not without long-term complications. All patients who undergo gastrectomy will have postoperative weight loss. Many will have metabolic complications including malabsorption, diarrhea, and/or “dumping syndrome.” Dieticians are often required to assist postsurgical patients in nutritional management. There are

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**Table 1: (Updated) Criteria for CDH1 molecular genetic testing.**

| Two or more cases of gastric cancer in a family, with at least one histologically confirmed diffuse gastric cancer (DGC) diagnosed before the age of 50. |
| Three or more confirmed cases of DGC in 1st or 2nd degree relatives, independent of age of onset. |
| An individual diagnosed with DGC before the age of 40. |
| An individual or family members diagnosed with DGC and lobular breast cancer, one being diagnosed before the age of 50. |
also secondary surgical complications such as esophageal strictures. A more thorough understanding of the long-term physical and psychological effects of this surgery will only emerge through followup of these relatively young HDGC patients. The current consensus guidelines have called for a central registry of HDGC patients who have undergone prophylactic gastrectomies [4]. This would provide essential prospective data regarding effects of the surgery as well as long-term followup regarding disease-free status.

Prophylactic gastrectomies for HDGC will most likely occur at major academic institutions in which specialized gastrointestinal pathologist are readily available. The gastric specimen is inked and fixed with lymph node retrieval as in any gastrectomy specimen. However, following adequate (overnight) fixation, these specimens should be photographed with mapped sampling occurring from all anatomical zones as well as submission of the entire surgical margins. If carcinoma is not identified, additional mapped sampling will be required. The focal intramucosal signet ring cells are generally identified on H & E morphology. A combination of cytokeratin (positive immunoreactivity) and E-Cadherin (negative or reduced immunoreactivity; see Figure 2(b)) can highlight these foci, especially in areas of pagetoid spread. Interestingly, in their study of eight total gastrectomy specimens done for germline CDH1 mutations, Rogers et al. found two cases which showed reversion of E-cadherin expression in foci of deeply invasive adenocarcinoma while the superficial signet ring cells cancer showed the expected loss or reduced E-cadherin expression [21]. Additional, nonspecific histologic features described in some cases of HDGC include foveolar hyperplasia with or without tufting, cytoplasmic vacuolization, and clustered histiocytes or vaguely granulomatous reactions occurring around ruptured glands [4, 18, 21].

The IGCLC also suggests that female carriers of the CDH1 mutation receive high-risk screening for lobular breast adenocarcinoma from the age of 35. Other cancers, such, signet ring cell carcinoma of the colon, have been proposed to be associated with HDGC. Colon cancer screening may therefore be recommended in HDGC patients who have a pedigree showing colon carcinoma presenting before the age of 40. This type of personalized heightened cancer screening emphasizes the need for thorough documentation of family histories as well as the importance of enrolling HDGC patient into cooperative registries for ongoing clinical research.

4. Conclusion

At least one third of all patients with HDGC have an autosomal dominantly inherited mutation of the CDH1 (E-Cadherin) gene. Penetration is high with a specific phenotype of diffuse, signet ring cell morphology. Pathologists should be aware of this phenotype of patchy clusters of intramucosal signet ring tumor cells associated with a pagetoid spread of individual signet ring cells (Figure 2), although not known to be entirely specific, this finding, especially in a young patient, may warrant a discussion with the treating clinician regarding referral to a genetic counselor. In patients known to have HDGC, a multidisciplinary approach, including genetic counselors, subspecialized pathologists, clinical researchers, dietitians, and experienced gastrointestinal surgeons, is essential in the management of these patients. Due to the insidious nature of early lesions in this disorder, endoscopy is not an adequate screening method. However, annual white light high-definition endoscopy with extensive biopsy sampling may provide a method of surveillance for those patients who are not good surgical candidates, who refuse more aggressive methods, or are carriers of mutations (e.g., missense) where clinical significance is less defined.

The prophylactic gastrectomy specimen of our patient known to carry a CDH1 mutation was found to have multifocal intramucosal signet ring cell adenocarcinoma despite normal endoscopic exam and biopsies. Previous publications, of at least three major groups, document the same finding in multiple HDGC families, making these risk-reducing gastrectomies simultaneously therapeutic. Currently there is no way of determining the latent period between the intramucosal CDH1 −/− adenocarcinoma and invasion into submucosa. However, it is important to note that our patient had at least two family members that died of HDGC at ages younger (19 and 35 years of age) than his age at the time of gastrectomy (36 years of age). Until better screening tests emerge, gastrectomy may be the only adequate means of lengthening survival in carriers of the CDH1 mutation.

References


Review Article

Molecular Aspects of \textit{H. pylori}-Related MALT Lymphoma

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\textit{Helicobacter pylori}-related extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue is a paradigm for malignancy arising in an inflammatory background. While the diagnosis of \textit{H. pylori} gastritis is often straightforward, distinction between severe gastritis and early lymphoma can be difficult and requires careful assessment of clinical findings in addition to histological features and immunohistochemical results. A number of cytogenetic abnormalities have been discovered in \textit{H. pylori}-related lymphomas and several have clinical importance, related to the responsiveness of lymphoma to \textit{H. pylori} eradication therapy, but routine molecular studies are not widely utilized. While molecular methods may be used in equivocal cases, a trial of conservative therapy is warranted given the propensity for these lymphomas to regress with eradication of the organism. Once therapy is initiated, care must be taken to avoid a premature assignment of disease refractoriness because complete response can take several months to more than a year. Cases truly refractory to \textit{H. pylori} eradication therapy may be treated with adjuvant chemoradiation with a high response rate.

1. Introduction

\textit{Helicobacter pylori} is a common pathogen and the most frequent cause of gastric and duodenal ulcers (Figure 1) [1, 2]. This Gram-negative, curved bacillus was first recognized as the cause of human disease by Marshall and Warren in the 1980s and has since been classified as a class I carcinogen, potentially leading to gastric adenocarcinoma and, more commonly, extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) [2–6]. Most \textit{H. pylori}-related MALT lymphomas arise in the stomach, but extragastric lymphomas may also be related to the organism, particularly in the duodenum [7]. The gastritis caused by \textit{H. pylori} is morphologically distinctive, with a band-like infiltrate of plasma cells and lymphocytes in the superficial gastric mucosa, typically in the antrum, accompanied by active (neutrophilic) inflammation in the mucus neck region of the mucosa, at the interface between the foveolae and glands. While \textit{H. pylori} gastritis is most often easily recognized, the dividing line between severe gastritis and early MALT lymphoma is frequently indistinct [6, 7].

Several cytogenetic abnormalities have been described in gastrointestinal (GI) MALT lymphoma, and inappropriate activation of the NF-kB pathway is a common thread [8–11]. While molecular assays for these abnormalities are available, guidelines for their routine use are not widely accepted, and the vast majority of cases of gastric as well as many extragastric, MALT lymphomas respond to conservative therapy aimed at eradication of infection [8, 12–17]. Some cytogenetic translocations seen in a subset of MALT lymphomas, however, are associated with resistance to antibiotic therapy. The routine use of molecular assays in diagnosis and prognosis of MALT lymphomas is controversial, and many cases are negative for any known cytogenetic abnormality [18–20].

This paper summarizes the current knowledge of cytogenetic abnormalities in GI MALT lymphoma, with particular attention to \textit{H. pylori}-related gastric lymphomas. Currently available molecular testing methods are discussed, followed by a practical approach to their use in diagnosis. Finally, recommendations for disease followup are offered, with an emphasis on the utility of conservative therapy and
avoidance of a premature determination of refractoriness to *H. pylori* eradication therapy.

### 2. MALT Lymphoma: A Review of Concepts

MALT lymphoma (Figure 2) is a low-grade B-cell lymphoma composed predominantly of small lymphocytes, first described around the same time as *H. pylori* by Isaacson and Wright [21]. Morphologically, the lymphoma cells may be centrocyte-like (with small nuclei and scant cytoplasm, resembling follicle center cells) or monocytoid (with ample pale cytoplasm and indented nuclei), often with admixed centroblast-like cells (large cells that may have prominent nucleoli) [6]. Monocytoid cells are fairly characteristic of MALT lymphoma, but individual cases are commonly a mixture of the three cell types, with centroblast-like large cells typically being individually scattered. Plasmacytic differentiation is common, and some cases are almost completely plasmacytic in appearance [6, 22]. While immunohistochemistry (IHC) is useful in diagnosis, there remains no specific IHC marker for MALT lymphoma [7, 18].

MALT lymphoma accounts for about 8% of all B-cell lymphomas and tends to occur in older individuals with a nearly equal sex distribution [22]. About 85% of all GI MALT lymphomas occur in the stomach, and isolated involvement of the small intestine is rare [7]. Conversely, about 25% of gastric MALT lymphomas are accompanied by involvement of other GI sites, and there is evidence that it is a truly systemic disease [6, 18, 23]. The vast majority of GI MALT lymphomas are thought to be related to *H. pylori* infection, which is believed to lead to malignant transformation via chronic antigenic stimulation resulting in the clonal expansion of subpopulations of B cells [18, 24–27]. The noninfected stomach is largely devoid of lymphoid tissue; *H. pylori* gastritis owes its appearance to the acquisition of a lymphoplasmacytic infiltrate in the gastric lamina propria, which may or may not be accompanied by B-cell nodules and even germinal centers [6, 7, 18]. This type of tissue is termed “acquired MALT” to distinguish it from “native MALT” of the type seen, for example, in the distal ileum, where germinal centers make up the Peyer’s patches.

Interestingly, while MALT lymphoma is a clonal B-cell neoplasm, the process of lymphomagenesis is believed to be driven by activated T cells [7, 27–29]. *H. pylori* strains expressing the CagA gene have been associated with significant morbidity, and this gene may play a role in lymphomagenesis [30]. The transformation of *H. pylori*-related MALT lymphoma to diffuse large B-cell lymphoma (DLBCL) has been postulated, although the exact mechanism of such a transformation remains obscure [6, 11, 18]. DLBCL is the most common GI lymphoma overall and the presence of a large cell component should be mentioned in the diagnosis when it occurs in association with MALT lymphomas [7, 22]. Sheets of large (centroblast-like) cells should prompt an unequivocal diagnosis of DLBCL, even when a recognizable low-grade MALT component is also present. Furthermore, such a low-grade component should be mentioned with the diagnosis of DLBCL when present. The former diagnosis of “high-grade MALT lymphoma” is no longer recognized and should not be used.

### 3. Cytogenetic Abnormalities in Gastrointestinal MALT Lymphoma

#### 3.1. t(11;18)(q21;q21) API2/MALT1

The t(11;18) translocation involves the fusion of the N-terminus of API2 (apoptosis
inhibitor-2) on chromosome 11 and the C-terminus of MALT1 (MALT lymphoma associated translocation) on chromosome 18. It is the most common translocation found in MALT lymphomas of the GI tract, involving up to 25% of gastric MALT lymphoma and 40%-60% of MALT lymphoma occurring in the small intestine [31, 32].

The presence of this translocation correlates with resistance to antibiotic therapy and MALT occurring without concomitant chronic active H. pylori gastritis, although it has been seen in specific strains of H. pylori infection [32, 33]. Cases with this translocation are more likely to have disseminated rather than stage I disease, but it is infrequently associated with diffuse large B-cell lymphoma [34, 35]. Some evidence suggests that plasmacytic morphology may not be seen in such cases [36].

Assays for the presence of t(11;18) are widely available. Methods include fluorescence in situ hybridization (FISH) or reverse transcription polymerase chain reaction (RT-PCR), both of which can be performed on fresh tissue or archival formalin-fixed paraffin-embedded tissue. FISH is more sensitive in the rare cases that do not involve the most common breakpoint. Dual color dual fusion FISH has been in use for the past ten years utilizing metaphase chromosomes or interphase nuclei [37]. In addition, break-apart MALT1 FISH probes are available, relatively easily interpreted in comparison to fusion probes, and may have the advantage of being useful in detecting MALT1 rearrangements in either t(11;18) or t(14;18) (see below) using one assay. IHC for BCL10 protein will show nuclear expression in many cases with the translocation; however, this is not specific and can be seen in t(1;14)(p22;q32) in association with the BCL10/IGH fusion [32]. The vast majority of cases with t(11;18) will show weak cytoplasmic MALT1 protein expression using IHC [38].

3.2. t(14;18)(q32;q21) IgH/MALT1. The t(14;18) translocation involves the immunoglobulin heavy chain gene (IgH) on chromosome 14 and MAL T1 on chromosome 18 which activates the NF-κB pathway [39]. This translocation may coexist with trisomies (3, 12, or 18). While the breakpoint on chromosome 18 is in the same region as that seen in follicular lymphoma, the gene involved is MAL T1 rather than BCL2; rare cases of MALT lymphoma have reportedly been associated with the IgH-BCL2 fusion [40]. While IgH/MALT1 is present in a large minority of MALT lymphomas, it occurs more commonly in non-GI sites. It has been reported, however, in unusual GI sites such as the liver [41].

Assays to detect t(14;18) include dual fusion FISH and PCR. IHC for MALT1 and BCL10 is highly sensitive, typically exhibiting strong, uniform cytoplasmic expression [38]; however, neither of these immunostains is specific for this translocation, as weak cytoplasmic staining for MALT1 and strong nuclear staining for BCL10 are characteristic of t(11;18). As these immunostains can be expressed in other translocations, they are not specific markers.

3.3. t(1;14)(p22;q32) BCL10/IGH. The t(1;14) translocation juxtaposes the BCL10 gene on chromosome 1 with the IgH gene on chromosome 14. A variant translocation involving lambda light chain on chromosome 2 has also been reported [42]. These translocations are present in a minority of MALT lymphoma (1%-3%); however, it is important as gastric MALT lymphoma with this translocation can also demonstrate antibiotic resistance [43]. It has not been reported in other non-Hodgkin lymphomas. As mentioned previously, IHC for BCL10 shows strong nuclear expression. MAL T1 is weakly expressed in the cytoplasm [38].

3.4. t(3;14)(q27;q32) BCL6/IGH and Other BCL6 Rearrangements. BCL6 translocations are involved in a small number of MALT lymphomas, approximately 1%-2% in one large study [44]. The translocation can involve the immunoglobulin heavy chain, light chains, or other partners. Of relevance in GI cases, it has been reported in cases of diffuse large B-cell lymphoma with concurrent MALT in the stomach [45].

BCL6 rearrangements can be identified by FISH using dual color break-apart probes. In a subset of BCL6 rearranged cases, IHC for BCL6 protein will show nuclear staining of the lymphocytes which can cause diagnostic confusion with follicular lymphoma [44]. Only 25%-30% of the cases with the translocation in this study showed BCL6 expression by IHC. The number of cases is small, and additional large studies are needed.

3.5. t(3;14)(p14.1;q32) FOXP1/IGH. This translocation can be seen in diffuse large B-cell lymphoma and MALT lymphomas (ocular, thyroid, and cutaneous) without the t(11;18) [46]; it has not been commonly associated with MALT lymphomas of the GI tract.

3.6. Trisomy 3 and 18. Chromosomal trisomies are commonly detected in gastrointestinal marginal zone lymphomas but are nonspecific. Trisomy 3q27 is the most common chromosomal abnormality in gastrointestinal lymphomas. In one large series, it was present in 50%-65% of low-grade MALT lymphomas from stomach and small intestine [47]; however, other series showed a lower prevalence [31, 48]. It occurs in both low-grade marginal zone lymphomas and DLBCL and is more common in patients with higher stage disease [49]. Trisomy 18 can be seen independently or in association with trisomy 3 and has also been correlated with more aggressive disease, especially in gastrointestinal lymphomas classified as diffuse large B-cell lymphoma [50]. Both trisomies can be detected using FISH with chromosomal enumeration probes, and break-apart probes for BCL6 and MAL T1 may also potentially identify these abnormalities.

4. Diagnosis of MALT Lymphoma and Use of Molecular Assays: A Pragmatic Approach

The distinction between severe H. pylori gastritis and early MALT lymphoma is often difficult and requires careful assessment of clinical findings as well as histomorphology and IHC. A carefully reasoned and evidence-based diagnosis
of an incipient MALT lymphoma may not be clinically helpful when the lack of endoscopic findings makes it impossible for the gastroenterologist to find an appropriate site for rebiopsy to assess the effectiveness of therapy. Thus, discussion with clinical colleagues and a conscientious search of the clinical and endoscopic record for lesions including masses, malignant-appearing ulcers, and diffusely thickened gastric folds are helpful in evaluating the patient for malignancy. In reality, the vast majority of gastric MALT lymphomas—possibly as many as 80% or more—will regress in time with conservative H. pylori eradication therapy, including cases that are organism negative by IHC [51]. Some evidence suggests that MALT lymphomas in locations outside the stomach may also regress with conservative therapy. As a result, erring on the side of diagnostic caution in the setting of dubious clinical findings is prudent.

Morphological features helpful in the diagnosis of MALT lymphoma include bona fide epithelial and mucosal injury, typified by the so-called “lymphoepithelial lesion”, a characteristic but nonspecific infiltration of epithelial structures by lymphoma cells (Figure 3) [6, 7, 52]. Care must be taken to avoid overinterpretation of such lesions, however, as they may also appear in benign settings including reactive lymphoid infiltrates and in crypts adjacent to normal Peyer’s patches. Reactive germinal centers, common in the deeper mucosa in H. pylori gastritis, may be colonized by lymphoma cells, with destruction of the mantle zone and the appearance of so-called “naked” follicles [6, 7]. In more extensive cases, the lymphoma can create mucosal ulcers and can infiltrate the muscularis mucosae, the submucosa, and even the muscularis propria. Muscularis mucosae infiltration and disruption can be a useful clue to the diagnosis in small biopsy specimens. While no specific IHC marker is available, an overabundance of B cells is present on CD20 stain, and aberrant coexpression of CD43 by neoplastic B-cells is found in up to 50% of cases in large series [6, 53]. In cases with extensive (or nearly complete) plasmacytic differentiation, IHC for kappa and lambda light chains can be extremely useful in highlighting a restricted plasma cell population, as such cases can closely mimic the intense plasma cell infiltrate of severe H. pylori gastritis. The standard application of light chain IHC, however, is not particularly helpful for determining the clonality of small lymphocytes, in our experience.

Studies of clonality can be useful in lymphoma diagnosis, but routine use of molecular studies such as heavy-chain gene rearrangement assays to aid in a determination of lymphocyte clonality in MALT lymphoma is not our practice. Clonal populations have been demonstrated in nonneoplastic H. pylori gastritis and, as noted earlier, equivocal cases are probably best treated conservatively as most will respond to H. pylori eradication [54, 55]. In addition, a significant number of MALT lymphomas may not exhibit detectable clonal IgH rearrangements [54, 56]. Furthermore, biopsy specimens are usually small and easily exhausted, putting the slides cut for adequate morphological and IHC analysis at a premium. Thus, the differential diagnosis of severe gastritis and incipient lymphoma is best made based on a combination of clinical information, histomorphology, and IHC.

Whether pretreatment analysis of molecular cytogenetics to determine the likelihood of treatment response is useful remains a topic of debate, although some reports indicate that it can be used in equivocal cases to help determine malignancy [36]. Reported cytogenetic abnormalities in gastric MALT lymphomas are summarized in Table 1.

Once the diagnosis of MALT lymphoma has been made and H. pylori eradication therapy initiated, the definition of treatment failure must be considered. While the majority of cases will respond to conservative therapy, the timing of rebiopsy for assessment of response is crucial in avoiding an inappropriate judgment of failure. Complete resolution of the lymphoid infiltrate typically takes several months, and periods as long as two years to complete resolution have been reported [14]. While it is typical practice in the setting of colonic adenomas to rebiopsy for assessment of complete polyp removal after a few weeks’ time to allow for mucosal healing, at least eight to twelve weeks between initiation of therapy and followup biopsy for MALT lymphoma is more prudent. Certainly, an assessment of failure is inappropriate before at least eight to twelve weeks have elapsed, as the lymphomatous infiltrate may not look substantively different in such a short time. Even at two to three months’ treatment duration (some observers suggest as long as a year), only a morphologically obvious worsening of the infiltrate or endoscopically visible increase in mass or lesion size should be considered a likely failure. In addition, molecular evidence of clonality by IgH gene rearrangement studies may persist for years after morphological remission is achieved, apparently without affecting patient outcome [14, 57]. Molecular analysis for t(11;18)(q21;q21) may be used in unresponsive cases, although the known association of this translocation with refractoriness to H. pylori eradication therapy may make such an assay unnecessary at this point in the disease course. Furthermore, there have been reports of antibiotic response
Table 1: Cytogenetic abnormalities in gastric MALT lymphomas, along with their reported frequencies, known clinical implications and available assays for their detection.

<table>
<thead>
<tr>
<th>Abnormality</th>
<th>% Gastric MALT</th>
<th>Clinical implications</th>
<th>Available assay(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(11;18)(q21;q21)</td>
<td>Up to 25%</td>
<td>Antibiotic resistance</td>
<td>FISH; RT-PCR; BCL10 IHC (nuclear)</td>
</tr>
<tr>
<td>t(14;18)(q32;q21)</td>
<td>Up to 5%</td>
<td>Unknown</td>
<td>FISH; PCR; MALT1 and BCL10 IHC (cytoplasmic/perinuclear)</td>
</tr>
<tr>
<td>t(1;14)(p22;q32)</td>
<td>Rare</td>
<td>Antibiotic resistance</td>
<td>BCL10 IHC (nuclear)</td>
</tr>
<tr>
<td>t(3;14)(q27;q32)</td>
<td>Rare</td>
<td>Reported in DLBCL with concurrent MALT</td>
<td>FISH (break-apart); BCL6 IHC (only 25%–30%)</td>
</tr>
<tr>
<td>Trisomies</td>
<td>5%–65%, depending on series</td>
<td>3q27 most common; associated with high-stage disease</td>
<td>FISH</td>
</tr>
</tbody>
</table>

in cases with this translocation [51, 58]. Thus, further study is needed to determine the utility of molecular assays in determining prognosis and directing therapy for patients refractory to conservative treatment [59].

Refractory cases may require more aggressive adjuvant therapy, including systemic chemotherapy and/or radiotherapy [12, 60]. MALT lymphomas have been found to be very sensitive to such systemic treatment [61, 62]. Cases with minimal residual mucosal disease, however, may be best managed by watchful waiting [63, 64]. Rarely, but particularly in cases that have transformed to DLBCL, surgery may be necessary to deal with complications such as intractable bleeding or perforation, and there is some discussion about whether surgical intervention should be reconsidered for primary therapy [65]. In addition to the presence of certain translocations described earlier, other factors associated with refractoriness to conservative therapy are transmural infiltration and transformation to DLBCL. A small number (around 10%) of cases will relapse, but this is typically associated with \textit{H. pylori} reinfecion [60].

To summarize, while the diagnosis of gastric MALT lymphoma and its definitive differentiation from severe \textit{H. pylori} gastritis can be difficult and are not amenable to a specific, algorithmic approach, our diagnostic methodology involves several touchstones. First, the simple presence of a lymphoplasmacytic infiltrate, with or without \textit{H. pylori} organisms, is insufficient for a diagnosis of MALT lymphoma and, indeed, is probably best regarded as \textit{H. pylori}-type gastritis and treated conservatively. We generally require microscopic evidence of significant mucosal disruption and injury—ideally accompanied by macroscopic features such as an endoscopically visible ulcer, mass, or thickened gastric folds—to raise any suspicion of lymphoma. In this setting, we perform an IHC panel that includes CD3, CD20, and CD43 to confirm an excess of CD20-positive B cells, possibly with the addition of kappa and lambda light chain stains if a significant plasmacytic component is suspected. aberrant coexpression of CD43 by the B cells adds further evidential weight to the diagnosis, but is only present in up to 50% of cases. We rarely, if ever, utilize molecular assays for either clonality or specific chromosomal abnormalities, as biopsy tissue is generally sparse and the vast majority of cases will respond to \textit{H. pylori} eradication therapy. For followup, we recommend rebiopsy after at least 12 weeks, with the expectation that the infiltrate at that point may not be markedly better, but should at least be no worse. We routinely compare follow-up biopsies to previous material and, given the known propensity for MALT lymphoma infiltrates to persist for several months to more than a year, we assiduously avoid an assignment of treatment-refractory status to a lymphoma unless it fails to appreciably improve after several months of therapy or recognizably worsens during therapy. For follow-up biopsies, we mention the status of the infiltrate in our reports, either in the diagnostic line or in a comment, such as "Stomach, biopsy: Residual MALT lymphoma, significantly improved from the prior biopsy on [date]."

5. Conclusion

Surgical pathologists commonly face the diagnosis of \textit{H. pylori} gastritis, and severe cases can be difficult to distinguish from \textit{H. pylori}-associated MALT lymphoma, particularly in the absence of suspicious clinical features. Happily, the large majority of such lymphoma cases respond to conservative antibiotic-based therapy for \textit{H. pylori} eradication. Several cytogenetic abnormalities, including chromosomal translocations and trisomies, have been described in MALT lymphoma, and two translocations \{t(11;18)(q21;q21) and t(1;14)(p22;q32)\} are associated with resistance to conservative treatment. The routine use of molecular studies, including those for clonality such as IgH gene rearrangement, in the diagnosis of MALT lymphoma is controversial, however, and a trial of conservative therapy is probably the best initial approach given the propensity for response to such treatment. Careful assessment of response is crucial, since lymphomas may take several months to more than a year to exhibit a complete resolution of the malignant lymphoid infiltrate. Thus, a premature declaration of treatment refractoriness should be avoided in order to prevent the inappropriate use of more aggressive adjuvant therapy.
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Review Article
HER-2/neu Testing and Therapy in Gastroesophageal Adenocarcinoma

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Despite ongoing advances in the treatment of gastroesophageal cancer, prognosis remains poor. The best promise to improve this poor survival is provided by new targeted agents. Of these, human epidermal growth factor receptor 2 (HER2) is currently in the spotlight. In this review, we provide an overview of recent developments in HER2 testing and results of clinical trials targeting HER2 in gastroesophageal adenocarcinoma. Based on the encouraging ToGA trial findings it is now expected that routine HER2 testing will be included in the diagnostic work-up of patients with advanced gastric cancer. With regard to this testing, overexpression of the HER2 protein seems to possess the best predictive properties. However, HER2 immunohistochemistry (IHC) is subject to assay and interobserver variability, so standardization and internal and external proficiency testing is an absolute prerequisite, especially as the IHC scoring system in gastric cancer is different from that of breast cancer. Further study is needed to investigate the clinical meaning of the significant heterogeneity observed in both gene amplification and protein overexpression in gastroesophageal cancer. Highly effective therapies for gastroesophageal cancer can only be accomplished by a multi-targeted approach, considering crosstalk between pathways and continuing to optimize chemotherapy.

1. Introduction

Despite ongoing advances in the treatment of gastroesophageal cancer, prognosis remains poor. Esophageal adenocarcinoma incidence has been rapidly increasing in Western countries during the past half century, especially in Caucasian males. This is believed to be attributable to the increased prevalence of gastroesophageal reflux disease and its major determinant, obesity [1], resulting in Barrett’s esophagus. Esophageal adenocarcinoma arising in Barrett’s esophagus has a poor prognosis with a 5-year relative survival of 10–20%. Gastric cancer affects about one million people per year and is the second leading cause of cancer-related mortality worldwide [2]. Gastric cancer is thought to result from a combination of environmental factors and accumulation of specific genetic alterations, and consequently, mainly affects older patients. Gastric cancer exists as two main histological types, diffuse and intestinal, as described by Lauren [3], and can be subdivided into proximal (cardia) and distal (corpus and pylorus) cancers. Interestingly, there seems to be a trend towards more proximally (cardiac) located gastric cancer. This distal to proximal shift is yet incompletely understood and seems to parallel the observed recent rise in incidence of Barrett’s esophagus. This fall in incidence in mid- and distal gastric cancer may be explained by the decline in Helicobacter pylori infection and associated atrophic gastritis. When a tumor is located at the gastroesophageal junction, it is often unknown whether the tumor is of esophageal or gastric origin. This group of cancers is therefore called “gastroesophageal junction cancers.” Surgery is the mainstay of treatment for resectable adenocarcinomas of stomach and esophagus, but recurrence rates are high even after radical resection. In Western countries, most patients are diagnosed at an advanced (unresectable) stage and, despite benefits of palliative radiotherapy and chemotherapy, survival of patients with advanced tumors remains poor (median survival 7–10 months) [4]. The best promise to improve this poor survival is provided by new agents.
acting against specific molecular targets. Of these, HER2 is currently in the spotlight. The aim of this paper is to provide an overview of recent developments in HER2 testing and results of clinical trials targeting HER2 in gastric and esophageal adenocarcinoma. Since most studies combine Barrett’s esophagus, gastroesophageal junction cancers, and gastric cancer, the term “gastroesophageal” is used to refer to this combined group.

2. Human Epidermal Growth Factor Receptor 2

HER-2/neu (HER2) is a proto-oncogene located on chromosome 17q21 and a member of the human epidermal growth factor receptor (EGFR) family. It encodes a 185 kD transmembrane tyrosine kinase receptor protein that, through dimerisation with other family members, regulates signal transduction in cellular processes including proliferation, differentiation, and cellular survival [5, 6]. Many studies have indicated a role of HER2 in the development of various types of human cancer. HER2 is amplified, and the expression of its receptor protein is increased in about 10–20% of breast carcinomas [7–11]. HER2 amplification and/or overexpression have also been observed in colon [12], bladder [13], ovarian [14], Fallopian tube [15], endometrial [16], lung [17], uterine cervix [18], head and neck [19], prostate [20], pancreatic [21], salivary gland [22], and esophageal [23] and gastric [24] carcinomas.

Patients with HER2-positive (amplification and/or overexpression) primary and metastatic breast tumors have increased survival rates when treated with trastuzumab (Herceptin), a recombinant humanized monoclonal anti-HER2 antibody [25, 26]. The efficacy of trastuzumab in breast cancer patients urged investigation into its antitumor activity in patients with other HER2-positive cancers, including gastroesophageal cancer. Furthermore, overexpression [27] and amplification of HER2 [28] have also been shown to correlate with poor prognosis and with resistance to conventional adjuvant chemotherapy and tamoxifen [29–33] in breast cancer. With the recognition of its prognostic, predictive, and therapeutic implications, assessment of HER2 status has now become of major importance in clinical practice for cancer patients.

2.1. Diagnostic Tests to Detect HER2 Amplification and Overexpression. Since the costs for trastuzumab therapy are high and side effects are significant, accurate selection of eligible patients for this therapy is crucial. Since 1998, trastuzumab has been used to treat more than 740,000 patients with HER2-positive breast cancer worldwide, so there is much to learn from the diagnostic methods used in the selection of breast cancer patients for this treatment. HER2 status is mainly assessed by immunohistochemistry (IHC) and chromogenic (CISH) or fluorescence in situ hybridization (FISH).

At present, the most common method to assess HER2 status is IHC [11] which is a routine technique available in most pathology laboratories to detect protein expression levels. Among HER2 IHC scoring systems for breast cancer, the HercepTest (Dako, Glostrup, Denmark) is frequently used to evaluate patterns of membranous immunoreactivity on tumor cells. The scoring system is based on intensity of reactivity, whether complete or incomplete and the percentage of reactive cells. Patterns are scored as IHC 0 (no staining or staining in <10% of tumor cells, negative), IHC 1+ (faint/barely perceptible incomplete membrane staining in >10% of tumor cells, negative), IHC 2+ (weak to moderate complete membrane staining in >10% of tumor cells, equivocal), or IHC 3+ (strong complete membrane staining in >10% (until 2007) or >30% (2007–now) of tumor cells, positive) [34]. Although staining and scoring methodology has been better standardized with the introduction of the Hercep Test than for most IHC assays, IHC is affected by poor tissue fixation, and there are still problems with reproducibility and interpretation of IHC assays [35–37], leading to both false negative and false positive IHC results.

In addition, in breast cancer, there is some evidence that testing for HER2 gene amplification provides better predictive information than IHC [38–41]. Gene amplification testing was traditionally mostly done by FISH. For FISH assessments, an HER2:CEP17 (centromeric probe 17) ratio of >2.2 is now used (≥2.0 before 2007) to define HER2 positivity (amplification), and ratios of 1.8–2.2 and <1.8 are used to define equivocal and negative categories, respectively [34].

Comparative studies of FISH and IHC have generally shown a high level of concordance in breast cancer [39, 42, 43]. Discordant results were mainly observed for tumors that were scored 2+ by IHC. However, pathologists have been reluctant to embrace routine FISH testing, because it is a difficult, expensive, and cumbersome technique that requires trained personnel which is not available in every pathology laboratory. Moreover, fluorescence fades upon storage, making it difficult to preserve the slides for future reference, and the fluorescent probes in the kits have a limited half life. Furthermore, detailed morphological features of the tumor are usually difficult to observe due to the required protein digestion and the fluorescent mode, and heterogeneity can be missed since spots are evaluated at ×100 magnification using oil immersion.

Chromogenic in situ hybridization (CISH) was introduced as an alternative for FISH in 2000 by Tanner et al. [44], using an immunoperoxidase reaction to detect specific DNA probes, which makes visualization possible with a conventional bright field microscope. Furthermore, similar to IHC, a permanent staining record is retained, and better morphologic examination is possible facilitating detection of heterogeneity. This is important in gastroesophageal cancer since higher rates of heterogeneity have been reported in gastric cancer (5%) compared to breast cancer (1.5%) [45]. CISH is also easier to interpret for pathologists who are not trained in fluorescence microscopy, and it is less expensive than FISH. In CISH scoring, the presence of large peroxidase-positive intranuclear clusters or ≥10 individual small signals in >50% of tumor cells (counted in at least 20 tumor cells) indicates HER2 positivity (amplification). The presence of small peroxidase-positive intranuclear clusters or 6–10 individual small signals indicates a low-level amplification,
and 5 or less individual small signals are scored as HER2-negative [34].

In several breast cancer studies, HER2 CISH correlated well with FISH and IHC [44, 46–51]. In gastric cancer, one study systematically analyzed the concordance between CISH and FISH assays and observed a perfect correlation in 128 samples [52]. However, one drawback of CISH assays is that amplification can only be assessed semiquantitatively. Therefore, detection of amplification by easier quantitative PCR techniques has been proposed as an alternative. One of the newly introduced techniques for detection of HER2 amplification in breast cancer is multiplex ligation-dependent probe amplification (MLPA) [53]. This technique determines relative copy numbers in a quantitative way and requires only minute quantities of small DNA fragments, which makes it very suitable for DNA isolated from paraffin-embedded material. In previous studies in breast cancer, excellent results were obtained with MLPA in comparison with IHC, CISH and FISH [10, 11, 51, 54, 55]. All currently available Food and Drug Administration approved or Clinical Laboratory Improvement Amendment validated HER2 tests have been recently summarized by Allison [56].

To eliminate discrepancies observed between IHC and FISH, Hofmann et al. [45] established an IHC scoring system specific for gastric cancer. In an international consensus meeting, modifications to the breast scoring system were made mainly based on the more frequent basolateral (incomplete) membrane staining and heterogeneity in gastric cancer. This new scoring system, illustrated in Table 1, has also been used to select patients for a clinical trial to evaluate trastuzumab efficacy and safety in HER2-positive advanced esophageal and gastric cancer [57]. A subsequent study validated these guidelines in terms of interlaboratory and interobserver consensus in a large series of gastric cancer and formulated additional specific recommendations [58]. For example, for reproducible intensity scoring, the degree of microscopic magnification (×-fold) at which membranous (linear intercellular) staining is clearly visible should be considered. Strong tumor HER2 IHC staining is usually already directly visible. In these cases, only low magnification (×2.5–5) is needed to confirm strong staining intensity. In any case where high magnification (×40) is required for unequivocal demonstration of membranous staining, the tumor is scored IHC 1+. The interobserver variation results within a ring-study prior and after application of the magnification rule clearly were in favor of such an approach over nonstandardized wording, for example, of “barely visible” for IHC 1+.

2.2. Relationship between HER2 Amplification and HER2 Overexpression. Nine studies (totalling 1,232 samples) examining the frequency of HER2 amplification in gastroesophageal cancer showed a mean HER2 positivity rate of 19.2% (range 7–43%) [45], which is similar to the reported percentage of protein overexpression.

In breast cancer, it is generally thought that HER2 overexpression is the direct result of gene amplification [59]. In esophageal and gastric cancer, concordance percentages between amplification and overexpression reported in literature range between 86.9 and 96.4% [60], as illustrated in Figure 1. Nevertheless, primary results from a very recent phase III trial (ToGA) containing >3,800 advanced esophageal and gastric cancer samples showed that the frequency of samples with amplification but without corresponding overexpression was high (23%) compared to that in breast cancer suggesting that FISH testing may be the more relevant procedure to conduct on these tumor specimens [61]. However, preliminary data from this same trial reported that patients with amplified tumors without overexpression (IHC 0 or 1+) did not show a substantial overall survival benefit from trastuzumab (HR 1.07, median overall survival 10.0 months versus 8.7 months) in contrast to patients with IHC 2+/FISH positive or IHC 3+ tumors (HR 0.65, median overall survival 16.0 months versus 11.8 months) [57], suggesting that measuring HER2 at the protein level should be the primary screening method for selecting gastroesophageal cancer patients for trastuzumab therapy. Final publication of these trial data needs to be awaited to draw firmer conclusions, and further research may be necessary to clarify these findings. The pattern seen in breast cancer, where amplification of HER2 leads to an overexpression of the protein, does not seem to have been fully confirmed in gastric cancer yet. If the observations in the ToGA trial are correct, this might be similar to what has been reported with another gene/protein relationship in breast cancer: topoisomerase II alpha (TOP2A). Unlike TOP2A, HER2 protein expression is not cell-cycle dependent, so other mechanisms (increased receptor degradation, transcriptional repression) may lay behind this discrepancy. Also, gastric cancer may have more

<table>
<thead>
<tr>
<th>Reactivity characteristics</th>
<th>Score/classification</th>
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<tbody>
<tr>
<td>No reactivity or membranous reactivity in &lt;10% of tumor cells</td>
<td>0/negative</td>
</tr>
<tr>
<td>Faint/barely perceptible membranous reactivity in &gt;10% of tumor cells</td>
<td>1+/negative</td>
</tr>
<tr>
<td>Weak to moderate complete or basolateral membranous reactivity in &gt;10% of tumor cells</td>
<td>2+/equivocal</td>
</tr>
<tr>
<td>Moderate to strong complete or basolateral membranous reactivity in &gt;10% of tumor cells</td>
<td>3+/positive</td>
</tr>
<tr>
<td>Biopsy (not surgery) samples with cohesive either IHC3+ and/or FISH+ clones (at least 5 cells) are considered positive irrespective of size, that is &lt;10% of tumor area</td>
<td>3+/positive</td>
</tr>
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FISH: fluorescence in situ hybridization; HER2: human epidermal growth factor receptor 2; IHC: immunohistochemistry.
inherent genomic instability than breast cancer resulting in more genes which are amplified as a bystander effect and not necessarily resulting in a functional increase of protein expression.

2.3. HER2 Expression in Gastroesophageal Cancer. Reported rates of HER2 overexpression in gastroesophageal cancer vary widely (2–45%) due to small sample sizes, differences in patient populations and methodological and scoring differences between studies [45, 62–67]. In addition, differences between HER2 overexpression in European and Asian/South-American populations (22–28% versus 3–15%, resp.) have been reported by some studies [68], but others have found these differences to be less substantial [69]. The largest data set of >3,800 advanced esophageal and gastric cancer samples found HER2 protein positivity rates of 23% [57, 68]. A recent review combining data from 24 studies (6,542 patients) calculated a weighted mean of 19% HER2 positivity [60]. In the few studies that reported separate HER2 positivity rates for gastroesophageal junction cancers and gastric cancer, HER2 positivity was higher in gastroesophageal junction cancer (24–35%) than in gastric cancer (9.5–21%) [65, 68, 70, 71].

2.4. HER2 in Esophageal Cancer. The data on HER2 overexpression in esophageal cancer are variable, with most studies showing HER2 overexpression in 9%–60% of cases, whereas other reports failed to observe HER2 expression [72]. The differences among reported overexpression rates might depend on stage of the disease, tumor histology (adenocarcinoma versus squamous cell carcinoma), methodology, and interpretation of IHC results. The relationship between HER2 expression and the prognosis of patients with esophageal cancer is not clear. It has been demonstrated that HER2 overexpression correlates with tumor invasion and lymph node metastasis, and thus indicates a poor prognosis [71, 73–75]. Studies that specifically analyzed HER2 expression and/or amplification in Barrett’s esophagus reported positivity rates of 38–50% and showed an association with progression from Barrett’s esophagus to dysplasia and adenocarcinoma [76–79]. A very small pilot study showed that trastuzumab treatment caused HER2 downregulation and increased apoptosis in patients with dysplasia and adenocarcinoma arising in Barrett’s esophagus [80].

2.5. HER2 in Gastric Cancer. Previous studies have shown that early-onset gastric cancer (presenting at the age of 45 years or younger) forms a small (<10%) [81] but distinct group of gastric cancers with a different molecular expression profile than conventional gastric cancer [82–84]. We recently showed that these younger patients show very low (<5%) HER2 amplification and overexpression frequencies (unpublished data). Common gastric tumors classified as intestinal type are more likely to be HER2-positive (16–34%) than diffuse (2–7%) or mixed (5–20%) types [65, 68, 70]. The reason for the selective overexpression of HER2 in intestinal-type gastric cancers is complex and needs further investigation. The association of HER2 with a specific type suggests that intestinal- and diffuse-type gastric cancers develop along different molecular pathways and supports earlier studies showing distinct patterns of genetic alterations in gastric cancers of differing histopathologic features [85]. Some similarities can be drawn with breast cancer: diffuse-type gastric cancers and lobular invasive breast carcinomas are both associated with E-cadherin loss, which is inversely correlated with HER2 amplification/overexpression which is more common in ductal invasive breast carcinomas and intestinal-type gastric cancers.

Although some studies have reported that HER2 amplification and overexpression are highly homogeneous within a tumor and between primary and metastatic gastric cancer [62], others have reported significant heterogeneity in both gene amplification and protein overexpression in individual cancers, even among IHC 3+ cancers [67, 86]. Some studies, including our own findings (unpublished data), showed homogeneous HER2 gene amplification but heterogeneous HER2 protein expression in certain samples, indicating that false negatives might arise when IHC is employed to predict trastuzumab response, especially when insufficient material is examined, such as in gastric biopsy specimens [52]. Generally, higher rates of HER2 heterogeneity have been reported in gastric cancer (5%) compared to breast cancer (1.5%) [45]. Chromosomal instability is probably one of the major causes of this heterogeneity. It needs to be shown whether patients with small cohesive HER2-positive clones show a different response to trastuzumab compared with patients with extended HER2-positive areas.

Although reports are conflicting, some studies have suggested that HER2-positive status in gastric cancer is associated with poor outcomes and aggressive disease [65, 70].

3. Preclinical and Clinical Data: Anti-HER2 Therapy

Several studies have indicated antitumor activity of trastuzumab and lapatinib in human gastric cancer cell lines (NCI-N87, 4-1ST, SMU-216, MKN-45P) or xenograft models which overexpress HER2 [65, 87–90]. In these preclinical studies, these targeted compounds have been shown to be effective both as single agents and in combination with chemotherapeutic agents that are widely used for the treatment of gastric cancer. The three-drug combination of capecitabine (Xeloda, Roche), cisplatin, and trastuzumab showed a remarkable tumor inhibitory effect in the NCI-N87 tumor xenograft model, and it is this drug combination that was also used in the ToGA trial. This trial was the first randomized, prospective, multicenter, phase III trial to study the efficacy and safety of first-line trastuzumab in HER2-positive advanced gastroesophageal cancer [60, 68]. The modest but clinically significant overall survival benefit indicated that trastuzumab is a new, effective, and well tolerated treatment for HER2-positive gastroesophageal cancer. In this trial, patients with gastroesophageal cancer (n = 3,807) were centrally tested for HER2 status by IHC and FISH (patients were eligible if their tumor samples were scored
as 3+ on IHC or if they were FISH positive (HER2:CEP17 ratio ≥2.0), and 22% were HER2 positive. HER2 positivity was higher in esophageal junction cancers (33%) than gastric cancer (21%), and tumors classified as intestinal type (32%) were significantly more likely to be HER2-positive than diffuse (only 6%) or mixed (20%) types. Median overall survival, the primary endpoint, was significantly prolonged in the trastuzumab plus chemotherapy arm when compared with chemotherapy alone (13.5 months versus 11.1 months; \( P = .0048 \)), representing a 26% reduction in the risk of death in the trastuzumab group (hazard ratio 0.74, confidence interval 0.60–0.91). The overall response rate was also significantly greater in the trastuzumab arm (47.3% versus 34.5%; \( P = .0017 \)). Safety profiles were similar in the two study groups with no unexpected adverse events being reported with the addition of trastuzumab to chemotherapy.

No clinical data with lapatinib are available so far, but several phase II studies are ongoing and even a phase III trial has been initiated. Compared to trastuzumab, lapatinib is a small molecule tyrosine kinase inhibitor (TKI) that targets both HER2 and epidermal growth factor receptor (EGFR/HER1) and can be administered orally. It would be interesting to see whether the dual action of lapatinib will provide additional benefit over trastuzumab, especially in view of the fact that EGFR also seems to be overexpressed in gastric cancer [91–93].

4. Changing Treatment of Esophageal/Gastric Cancer

Despite advances in clinical diagnostics, surgical techniques, chemotherapy, and radiotherapy regimens, prognosis of gastric cancer remains poor, and novel treatment options as well as predictors of treatment response are urgently needed. Van Cutsem et al. presented preliminary results of the ToGA study at the 2009 ASCO Annual Meeting [94]. Although data from this trial should be considered encouraging and a major step forward in the treatment of advanced gastric cancer, some important considerations should be made. Firstly, the absolute benefit in response to trastuzumab addition to chemotherapy was 12.8%, indicating that—as in breast cancer—there is also resistance to trastuzumab even among HER2-positive selected patients. A better understanding of HER but also of other signalling pathways such as the Wnt and TGFβ pathways is therefore crucial. A combination of targeted agents, which ideally target different “crosstalk” pathways, would theoretically lead to highly effective therapies [95]. Secondly, although trastuzumab efficacy is likely in the adjuvant setting, trastuzumab use in early gastric cancer requires the completion of new adjuvant phase III trials. Thirdly, although some studies have reported that HER2 amplification and overexpression are highly homogeneous within tumors and between primary and metastatic gastric
cancer [62], others have reported significant heterogeneity in both gene amplification and protein overexpression in individual cancers, even among IHC 3+ cancers [67, 86]. This could impede predicting treatment response and thus selecting the right patients for treatment.

Future directions of research in HER2-positive gastroesophageal cancer should focus on the evaluation of novel antibodies (such as pertuzumab, a dimerization inhibitor, and T-DM1, a drug that combines trastuzumab with a linked chemotherapy agent called maytansine), irreversible tyrosine kinase inhibitors (such as neratinib and BIBW 2992, both dual EGFR-HER2 inhibitors), and inhibitors of HER2-related downstream signaling (such as mammalian target of rapamycin (mTOR), heat shock protein 90 (Hsp90), and PI3K/Akt) and of receptor crosstalk (such as other HER family members, vascular endothelial growth factor receptor (VEGFR), and insulin-like growth factor receptor (IGFRI)).

In the latter category, some promising targeted agents have already been investigated in gastroesophageal cancer. Molecular interactions between HER2 and other members of its family (HER1 or EGFR, HER3 and HER4) have led to the development of new targeted therapies such as the anti-EGFR monoclonal antibody cetuximab, the anti-EGFR oral small molecule tyrosine kinase inhibitors erlotinib and gefitinib, and the dual EGFR-HER2 tyrosine kinase inhibitor lapatinib [96]. Cetuximab has undergone more extensive evaluation in gastroesophageal cancers than any other targeted agent in the locally advanced setting as well as in the first-line metastatic setting, the second-line setting, and beyond. Unfortunately, most of these trial results have only been published in abstract form, and final publication is eagerly awaited. Cetuximab shows promise in the treatment of esophageal and gastric cancers in the locally advanced as well as in the first-line metastatic setting. Both erlotinib and gefitinib have very little single-agent activity in the first- and second-line settings in gastroesophageal cancers. Lapatinib has shown only modest results in the very few studies evaluating its activity in the metastatic setting. However, despite these modest results, the phase III LOGIC trial is currently evaluating the combination of capecitabine/oxaliplatin with or without lapatinib as first-line therapy in HER2-positive locally advanced, unresectable or metastatic gastroesophageal cancer.

HER2 has also been shown to communicate with VEGFR, and studies in breast cancer have shown a synergistic interaction with the combination of trastuzumab and a VEGFR tyrosine kinase inhibitor [97]. Therapies directed against VEGF(R) are the focus of ongoing research in many malignancies including gastroesophageal cancer [98, 99]. Bevacizumab, a monoclonal anti-VEGF antibody, has been investigated in the locally advanced and metastatic first- and second-line setting with encouraging phase II results [96]. A confirmatory international phase III trial of capecitabine/cisplatin with or without bevacizumab in advanced gastric cancer is currently underway. Sunitinib and sorafenib, both multitargeted (among which VEGFR) tyrosine kinase inhibitors, have been tested in metastatic gastroesophageal cancers with promising preliminary results [96].

5. Conclusion and Future Directions

Based on the encouraging ToGA trial findings, it is now expected that routine HER2 testing will be included in the diagnostic work-up of patients with advanced gastric cancer. With regard to this testing, overexpression of the HER2 protein seems to possess the best predictive value. However, HER2 IHC is subject to assay and interobserver variability; so standardization and internal and external proficiency testing is an absolute prerequisite, especially because the IHC scoring system in gastric cancer is different from that of breast cancer.

As in breast cancer, trastuzumab research will now most probably move into the adjuvant setting. The combined treatment of chemotherapy and trastuzumab may also be beneficial in decreasing the recurrence of the disease after resection of the tumor.

Given the high degree of primary and acquired resistance to trastuzumab therapy and bearing in mind that most patients with gastroesophageal cancer are HER2-negative, highly effective therapies can only be accomplished by a multitargeted approach, considering crosstalk between pathways and continuing to optimize chemotherapy.

Targeted agents will likely gain an increasingly important role in the treatment of patients with esophageal and gastric cancer in the near future, but how big a part they will play is unclear at this point. Lessons learnt over the past decades in breast cancer can help us maximize therapy benefit.

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