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The Molecular Biology of Adenoid Cystic Carcinoma

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Abstract

Background—Adenoid cystic carcinoma (ACC) is an unusual salivary gland malignancy that remains poorly understood. Standard treatment, including surgery with postoperative radiation therapy have attained reasonable local control rates, but the propensity for distant metastases has limited any improvement in survival over time. Our understanding of the molecular mechanisms driving adenoid cystic carcinoma is quite rudimentary, due to the infrequent nature of its occurrence.

Methods—An extensive literature review was performed on salivary gland adenoid cystic carcinoma and basic science research findings.

Results—This review highlights many findings that are emerging about the carcinogenesis of ACC including cytogenetics, tumor suppressor genes, oncogenes, epigenetic alterations, mitochondrial alterations, and biomarker studies.

Conclusions—While there have been many discoveries, much still remains unknown about this rare malignancy.

Keywords

molecular; genetics; DNA; adenoid cystic carcinoma

INTRODUCTION

Salivary gland adenoid cystic carcinoma (ACC) is a rare cancer, comprising only a small percentage of all head and neck malignancies. ACC is characterized by: 1) a propensity for indolent, yet progressive local growth, 2) aggressive histologic features such as perineural invasion, 3) favorable local control rates, 4) a 40% incidence of distant metastasis yet a very low incidence of regional metastasis, and 5) the possibility of stable metastasis whereby patients may live 10–15 years despite having metastatic disease[(1)]. The mainstay of treatment has not changed over many years, and includes local resection with the addition of

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radiation therapy. Chemotherapy has been used in isolated cases, but has not been proven to make a significant difference in local control or overall survival.

While these paradoxical features make ACC an interesting tumor model to study, it has not received much research focus, likely due to its rarity. We have reached a relative impasse with respect to the treatments offered, and because little is known about the molecular pathways underlying ACC, specific targeted agents or chemotherapy have not often been delivered with a mechanistic rationale.

The goal of this review is to discuss the molecular biology of ACC and to highlight the areas in which we have made some headway. By building upon this foundation and continuing to explore the molecular underpinnings of ACC, it is our hope that discoveries can be made that will greatly influence the care and outcomes of patients with this disease.

I. CYTOGENETICS

In recent years, cytogenetic studies on adenoid ACC have evolved substantially. The advancement of genetic methods has evolved, from karyotype G-banding to fluorescent in situ hybridization (FISH), comparative genomic hybridization (CGH) and microarray, to microsatellite PCR and loss of heterozygosity (LOH) analysis. Using these methods, a more complete and accurate picture of chromosomal abnormalities involved in ACC has begun to emerge (*see* Table 1). A better understanding of the cytogenetic profile of ACC not only provides potential mechanisms for pathogenesis, but also points to potential genes and regions of interest that can be further studied using other gene-specific methods.

a. G-banding

One of the first cytogenetic works done on adenoid cystic carcinoma (ACC) was reported by Sandros et al [(2)]. This study reviewed a large sample of 189 benign and malignant salivary gland tumors within the span of 10 years. However, due to the relatively rare occurrences of ACC, there were only 11 cases of ACC among the 189 tumors studied. In addition, the only cytogenetic analysis available at the time was G-banded stemline karyotyping. However, even with the restricted resolution of G-banding, chromosome 6q deletion was observed in more than 50% of the cases. Specifically, the breakpoints were clustered within the 6q22–25 region, causing a minimal common deletion of at least 6q25-qter [(3)].

The two proposed principal mechanisms by which 6q deletion could lead to tumorigenesis was through activation of an oncogene or loss of a tumor suppressor gene. Indeed, the oncogenes, c-*ros*, c-*syn*, and c-*myb* have been mapped to this region [(4)]. However, the wide variation in breakpoints on 6q suggested that the coincidental activation of these oncogenes was a less likely mechanism. It was thus postulated that a tumor suppressor residing in this region is lost during the deletion event. This can occur by a loss in the expression of the normal dominant phenotype or through the unmasking of a recessively mutated gene on the remaining chromosome 6 homologue. Moreover, these authors reported clear differences in the nature of chromosomal abnormalities between benign and malignant salivary gland tumors—that reciprocal translocations were more common in adenomas whereas deletions were more prevalent in malignant tumors [(2)].

b. Fluorescent in situ Hybridization

The next important stage in the evolution of detecting chromosomal abnormalities in ACC was the development of fluorescent in situ hybridization (FISH). FISH has been a useful technique not only in the confirmation of findings from band karyotyping [(5)] but also in refining these results [(6)]. For example, when the loss of 6q in ACC was re-evaluated by FISH and multicolor combined binary ratio labeling FISH (COBRA-FISH), this deletion

was found to have been misinterpreted by banding studies [6]. Specifically, Jin demonstrated that the distal 6q "deletions" were actually a result of seemingly balanced translocations between the long arm of chromosome 6 and the short arm of chromosome 9 (t(6;9)(q21–24;p13–23)), and actually, this was a more common event than previously suggested from karyotyping results. These findings have improved the precision of G-banding study observations made in earlier years and they were further confirmed by larger-scale studies [7].

Recently, advances in the FISH technique have allowed us to examine the expression of specific genes in the context of their location on the chromosome. For example, the expression of EGFR and HER2, two genes that have been implicated in non-small cell lung cancer and squamous cell carcinoma of the head and neck, have been examined using this technique. Vidal et al. [8] looked at the overexpression of these two genes using FISH in tumor specimens from patients with ACC treated with Lapatinib. Although they found that no gene amplifications were detected in ACC samples, patients who had either low or high HER2/chromosome-specific centromeric enumeration probe (CEP) 17 ratios had a prolonged progression-free time as compared to those with moderate ratios. This example illustrates that FISH can be a useful tool in identifying possible prognostic markers in ACC.

The most novel and recent finding using FISH was the recurrent fusion of *MYB* oncogene and *NFIB* transcription factor gene in ACC [9]. Using FISH, Persson et al. demonstrated that the t(6;9)(q22–23;p23–24) translocation led to the consistent fusion of *MYB* exon 14 to the last coding exon of *NFIB*. This event then caused the deletion of *MYB* exon 15 and the 3'-UTR which contains several regulatory regions. The loss of *MYB* regulation may have also led to overactivation of critical *MYB* targets that include genes involved in apoptosis, cell, cycle control, cell growth, and cell adhesion[9]. In an independent study with a larger cohort of ACC samples, the overexpression of *MYB* was confirmed in the majority of ACCs, although fusion gene *MYB-NFIB* was found in only 28% of primary and 35% of metastatic ACCs [10]. Most recently, West et al. demonstrated with FISH that the *MYB-NFIB* translocation was specific for ACC only and not present in other types of salivary tumors [11]. Moreover, ACC tumors with this translocation are possibly associated with higher local relapse rates, though the number of cases in this study was not sufficient to reach statistical significance. These studies on the *MYB-NFIB* translocation are some of the first to use FISH to elucidate a mechanism for ACC pathogenesis and provide a potential therapeutic target.

Overall, these studies show that FISH is a valuable tool to study chromosomal abnormalities and to detect amplification of entire regions or a few select genes. However, a limitation of this technique lies in its inability to detect small changes in gene amplification, especially those unrelated to chromosomal breakages. Newer techniques such as comparative genomic hybridization and microarrays reach higher levels of sensitivity in detecting abnormal expression levels. They also allow for the relatively rapid analysis of multiple sets of genes, thereby providing a more comprehensive picture of genetic abnormalities present in ACC.

c. Comparative Genomic Hybridization and Microarray

Findings via comparative genomic hybridization (CGH) have not only again confirmed the aforementioned chromosome 6q abnormalities by showing DNA copy number losses in this region [12, 13] but have also unraveled many additional ACC-related cytogenetic abnormalities. Novel CGH findings include DNA copy number losses at chromosome 12q12-q13 [13] and 1p32–36 [14], and gains at chromosome 22q12-q13 [15] [12], 8 [14], 16p [12], 17q [12] and 18 [14].

Microarray techniques have also helped refine earlier findings by determining expression levels of specific genes to develop a complex ACC profile. For example, Vekony et al. used array-based CGH to confirm several abnormalities discussed above including chromosome 6 and 9 translocations, 12q12–13 losses and 16p, 17q, and 22q13 gains. Within these abnormalities, they showed that *PDGF*, a mesenchymal cell growth factor located on 22q13 was increased in ~40% of ACC's. They also found several additional gained regions on chromosome 9q, 11q, and 19q that harbor numerous fibroblast growth factors and their receptors, suggesting that the increase in expression of these genes may be important in ACC development [16].

A more extensive characterization of the genetic profile of ACC was achieved by Frierson et al. [17] and Patel et al. [18] using oligonucleotide microarray analysis. Their findings indicate that ACC tumors are derived from differentiated salivary gland tissue that undergoes dedifferentiation and begins to re-express genes associated with early development. Of note, tumors showed high expression levels of the transcription factors *SOX4* and *AP-2γ*, and genes of the *Wnt/B-catenin* pathway [17]. They also significantly overexpressed genes involved in morphogenesis, muscle development, neurogenesis, and proliferation [18]. These data suggest that a transition from a mature salivary gland gene profile to a cancer-associated gene profile underlies ACC pathogenesis.

d. Microsatellite Marker and LOH analysis

A technique that has been frequently used to confirm and augment findings from CGH and microarray is microsatellite loss of heterozygosity (LOH) analysis [19–21]. Using microsatellite marker mapping, LOH analysis has also helped identify regions containing tumor suppressor genes lost in ACC.

Findings from these studies show that major regions of loss include 12q13.11-q13.3, 12q24.32-q24.33 [20] and 6q24.1-q25.1 [21], regions previously described using other methods. Additionally, these LOH analyses were linked to microarrays to identify downregulated genes within these regions. Several regulatory genes such as *HOXC5, ACVRL1, and AQP5* located on 12q12-q13, and a candidate tumor suppressor gene *DUSP6* located on a minor consensus deletion site on 12q23.3, showed significantly decreased expression in tumor samples [20]. Although two other tumor suppressor genes, *PLAG1* and *LATS 1*, were linked to the 6p24-q25 region, their expression levels in tumors were similar to normal, and their potential role in ACC pathogenesis was eliminated [21].

LOH studies have shown that 6p and 12q are "hotspot" regions of deletion, but it is worthy to note that there is no consensus region of loss [19]. Also, the number of genomic alterations in ACC is relatively low compared to other salivary gland tumor types. This implies that other mechanisms of oncogenesis that cannot be detected through cytogenetic methods, such as gene mutations or epigenetic regulation, may be predominant in ACC initiation, and they should be further investigated using other techniques.

II. ONCOGENES

From the results of cytogenetic studies, it is evident that the molecular mechanisms underlying tumorigenesis in ACC are complex and not well understood. However, based on what we know from other types of tumors, activation of cell-survival pathways with concomitant suppression of the pro-apoptotic machinery is an important step in pathogenesis. Naturally, this is believed to occur in ACC as well. A few notable cell-cycle and apoptosis regulators are discussed here along with some other oncogenes that have been shown to play a role in ACC pathogenesis (*see* Table 2):

a. Developmental Proteins

As previously mentioned, cytogenetic methods have showed that ACC may be derived from differentiated salivary gland tissue that reverts to an early developmental gene profile [17, 18]. Specifically, *sox4* and *c-kit* are two significant developmental proteins that have been attributed to ACC. Additionally, in recent cytogenetic studies, a novel role for the *MYB* proto-oncogene in ACC has also been identified.

Sox 4—One of the most highly over-expressed genes in ACC is Sry-related high mobility group (HMG) box 4 (*Sox4*) [17]. Originally identified as a transcription factor belonging to the HMG box superfamily of DNA binding proteins [22], *Sox4* has been reported to be over-expressed in a diverse array of human malignancies, but despite its upregulation in diseased states, the exact role *Sox4* plays in disease genesis and development is still being explored.

Sox4 is normally required for proper development of the cardiac outflow tract, pro-B-lymphocyte differentiation, and CNS development during embryogenesis [23, 24]. In ACC, *Sox4* seems to be important for the regulation of cell survival, proliferation, and apoptosis—knock-down of its expression using siRNA in an ACC-derived cell line decreased cell survival and enhanced apoptosis [25]. Of note, the validity of ACC-derived cell lines has been challenged [26] and thus any results with these cell lines should be interpreted cautiously. Interestingly, characterization of downstream targets of *Sox4* in metastatic hepatic cancer cells revealed genes important for cell migration, suggesting that it plays a role in metastasis [27]. It would be particularly pertinent to determine if *Sox4* plays a similar part in ACC, as one of the hallmarks of ACC is the formation of distant metastases.

c-kit—Another protein implicated for its potential role in malignant transformation in ACC is the transmembrane tyrosine kinase receptor *c-kit*. Structurally related to platelet-derived growth factor/colony stimulating factor-1 receptors [28], *c-kit* is activated by binding to stem cell factor (alternatively known as mast cell growth factor, steel factor), and promotes cell growth and differentiation [29]. *C-kit* activation is important for a variety of normal physiologic process, including hematopoiesis, spermatogenesis and growth and migration of melanocytes [30, 31]. However, it has also been documented in many human malignancies.

In ACC, *c-kit* expression has been shown to correlate with tumor grade [32], but the precise mechanism underlying c-kit activation has been elusive. Somatic mutations of c-*kit* have been identified in certain gastrointestinal stromal tumors, mast cell neoplasms and seminomas [33–35], but most studies have not shown these to be present in salivary ACC [28, 32]. Only one recent study has shown *c-kit* activating point mutations in exon 11 and less frequently in exons 9,13, and 17 [36] though there is current speculation that these supposed mutations are actually technical artifacts in relation to the authors' use of formalin-fixed, paraffin-embedded tissue [37]. Interestingly, copy number gains of the *c-kit* gene has been found in a small number of ACC cases [38], suggesting an alternative mechanism for *c-kit* upregulation in a subset of ACC patients. Regardless of the mechanism of upregulation, clinical trials looking at the effects of *c-kit* inhibitors in patients have been thus far disappointing [39].

In a recent study, a novel *c-kit* associated factor called *Slug* has also been implicated in ACC [40]. Zinc-finger transcription factor *Slug* (Snai2) is an important mediator of epithelial-mesenchymal transitions and has been associated with metastasis and poor prognosis in a number of different tumors [40]. Recently, *Slug* has been shown to contribute biologic specificity to the *SCF/c-kit* signaling pathway [41] and to control cell migration abilities [42]. In their study, Tang et al. showed that in ACC tumors, *c-kit* expression was correlated with *Slug* expression and that both were associated with increased TNM stage, perineural invasion, local regional recurrence and distant metastasis [40]. These findings

provide clues to the pathway by which *c-kit* can contribute to ACC pathogenesis and provide areas for further research.

MYB—*MYB* is a proto-oncogene for which a fusion transcript has been identified using cytogenetic methods [9]. However, due to small size of the cohort (n=12) in which the *MYB-NFIB* fusion was discovered, a more comprehensive study was done to identify the incidence of the fusion transcript in a larger sample size that included both ACC and non-ACC salivary gland tumors (n=123) (*see* Table 3). Using RT-PCR and immunohistochemical techniques, Mitani et al [10] showed that various *MYB-NFIB* fusion transcripts were present in 28% of primary and 35% of metastatic ACCs, but not in any other tumor types analyzed. *MYB* was also overexpressed in the majority of ACC samples, and significantly higher expression levels were found in the samples carrying fusion transcripts. Overall, this study shows that the *MYB-NFIB* fusion transcript is present in a specific subset of ACC tumors and is related to *MYB* overexpression. In a study of an additional 37 ACC tumors, West et al. also confirmed the findings of the *MYB-NFIB* translocation being specific to ACC [11]. However, they made an interesting point of showing that *MYB* immunostaining is confined to the basal cell component, though the translocation is present in all cells, suggesting that there are intact regulatory mechanisms in the neoplastic cells that can regulate levels of the fusion protein. These recent studies greatly solidify a role for *MYB* in ACC pathogenesis, and they confirm that *MYB* may be a good target for future therapeutics.

b. Growth Factors

Another group of genes that have been implicated in tumorigenesis in ACC is family of growth factors and their receptors. These include epidermal growth factors and receptors (EGFs and EGFRs), vascular endothelial growth factors and receptors (VEGFs and VEGFRs), and nerve growth factors (NGFs) [43–45].

EGF/EGFR—In the family of *EGFRs*, *EGFR* (*ErbB1*) is found to be over-expressed in ACC [45]. While expression of the proto-oncogene *HER2* (*ErBb2*) seems to be a good marker for salivary duct carcinomas [46], its expression in ACC has been controversial [43, 46, 47].

Ligands of *EGFRs* have been demonstrated to promote cell survival, proliferation, angiogenesis, and metastasis in diseased states [48]. Most recently, overexpression of epiregulin, a novel member of the *EGF* family, was shown to promote in vitro migration and invasion in a low metastatic rate cell line [49]. The effects of epiregulin are believed to be mediated through activation of *ERK1/2*, *Akt* and *Cox 2* [49], but other distinct cell signaling pathways have been shown to be activated by other *EGF's*. For example, the classic *EGFR* ligands, epidermal growth factor (*EGF*) and transforming growth factor-α (*TGF- α*), have been shown to promote oncogenesis via the *Ras-Raf-MAP* kinase pathway and the *Jak/Stat*-protein kinase C pathway (reviewed in [50]).

Interestingly, in squamous cell carcinomas of the head and neck, *EGFR* and *TGF-α* levels are positively correlated with decreased patient survival [51]. However, in malignant salivary gland tumors including ACC, *EGFR* expression levels were correlated with tumor histological grade, but not patient prognosis [52]. This suggests that *EGFR* may be involved in ACC pathogenesis, but the relationship is complex and requires further investigation.

VEGF/VEGFR—*VEGF* has long been recognized as an important angiogenic signal in many different human malignancies [53, 54], but its specific role in ACC is only recently being explored. *VEGF* expression has been shown to be regulated by the *NF-kB* signaling

pathway, a pathway responsible for enhancing endothelial cell motility [55]. Several studies have found up-regulated *VEGF* expression in ACC tumors with enhanced microvessel density [56] [57], and in some, it served as a significant prognosticator of survival outcome [56, 58]. Remarkably, administration of a dual inhibitor of *EGFR* and *VEGFR* to mice with established parotid gland ACC inhibited tumor growth and prevented lung metastasis [59] further augmenting the importance of the growth factors in ACC disease.

BDNF and NGF—A frequent finding in ACC is perineural invasion. This common complication can make surgical resection difficult, leading to a poor prognosis [60]. Evidence suggest roles for brain-derived neurotrophic factor (*BDNF*) and nerve growth factor (*NGF*), two secreted proteins belonging to the larger family of neurotrophins, in facilitating perineural invasion. Both *BDNF* and *NGF*, with its high affinity receptor TrkA, have been positively identified in ACC specimens [44, 61].

c. Signaling Molecules

Wnt/β-catenin—Mutations in components of the canonical *Wnt/β-catenin* signaling pathway commonly characterize various neoplasms [62] and have recently been implicated in ACC [63] [64]. In one study, 7 of 20 ACC specimens examined contained a mutation in at least one of three genes involved in the pathway: *CTNNB1*, *AXIN1*, and *APC* [63]. *CTNNB1* codes for β-*catenin*, a transcription factor that relocates from the cytoplasm into the nucleus in response to *Wnt* ligand binding. Once in the nucleus, it turns on genes important for cell growth and proliferation such as *cyclin D1* and *c-Myc* [65, 66]. Conversely, *AXIN1* and *APC* code for cytoplasmic proteins that complex together to sequester β-*catenin*, allowing for its eventual degradation by proteosomal machinery. Accordingly, mutations in any of the three genes can potentially contribute to tumorigenesis and disease progression by inappropriate activation of β-*catenin* either directly or through inactivation of its sequestration complex.

Not only differential expression, but also aberrant distribution of β-*catenin* can have pathological effects. Zhou et al. showed that reduced membranous expression of β-*catenin*, by itself, can be associated with ACC metastasis [67].

Recently, additional studies have identified other components of the *Wnt* pathway that are aberrantly expressed in ACC. For example, the *WNT* inhibitory factor 1 (*WIF1*), is known to be involved in a subset of salivary gland tumors, including ACC [64]. Interestingly, in this disease subset, a chromosomal translocation fuses WIF1 with *HMGA2*, resulting in loss of function of *WIF1*. *WIF1* normally behaves as a *Wnt* receptor antagonist, binding directly to *Wnt* to prevent receptor-ligand interaction and *Wnt* activation [68], Identification of the loss of *WIF1* function in ACC further stresses the importance of the *Wnt* pathway in its potential to contribute to disease in ACC.

Galectin-3 and *cyclin D1* are also *Wnt/β-catenin* associated proteins that have been recently shown to be important in ACC [69]. *Galectin-3* is a key regulator of the *Wnt/β-catenin* pathway [70] and its expression has been associated with more aggressive disease in many other types of malignancies [71]. In ACC, *Galectin-3* positivity is associated with regional and distant metastasis [72], and nuclear expression of *Galectin-3* in ACC may be related to a more aggressive tumor phenotype [69]. Additionally, *cyclin D1*, a crucial regulator of cell cycle progression and main product of the *Wnt/β-catenin* pathway has been shown to be overexpressed in a number of ACC samples [67, 69, 73].

Although a likely mechanism of ACC pathogenesis involving these factors is the dysregulation of the *Wnt* pathway due to loss of *WIF1* and/or gain of *Galectin-3*, causing an

increase in *cyclin D1* as an endpoint, studies aiming to elucidate this exact mechanism have shown that it may be more complicated and requires further study [69, 74].

III. TUMOR SUPPRESSOR GENES

Functional loss of tumor suppressor genes (TSGs) has been shown to play an important role in the tumorigenesis of many human cancers. First demonstrated in colon adenocarcinoma, alterations or loss of TSGs have since been confirmed in many other tumor types. TSGs require inactivation of both alleles, most often by point mutation and deletion or chromosomal rearrangement. Johns et al. found that allelic losses occurred frequently in ACC [75]; thus, loss of relevant TSGs likely contributes to carcinogenesis. This has been confirmed by studies looking at expressions and potential roles of several candidate TSGs in ACC tumors (*see* Table 4).

a. Cell Cycle Regulatory Proteins

p53—*P53*, located on chromosome 17p13, is the most commonly mutated gene identified in human neoplasms. If DNA damage occurs, *p53* accumulates within the nucleus and causes G1 arrest via the induction of *p21* protein, a potent inhibitor of cyclin-dependent kinases. If repair is unsuccessful, *p53* triggers apoptosis [76] [77] [78]. Cells harboring mutations in *p53* therefore will allow damaged DNA to replicate and are unable to induce apoptosis. Gene mutations may then accrue, leading to tumor formation and progression. Functional loss of *p53* has been demonstrated in many human cancers and is considered to play a critical role in malignant transformation [79, 80]. Mutations in *p53* have also been associated with more aggressive disease in a number of human cancers [81].

Most *p53* alterations are missense mutations that result in an inactivated protein that accumulates in the nucleus and can be detected by immunohistochemistry. Therefore, increased expression of the *p53* protein product is generally correlated with mutations in the gene. Rates of *p53* expression in ACC vary by report, from 0–80% of tumors [82–88]. These discrepancies may be in large part due to differences in sensitivity of *p53* staining. However, despite the differences in reported expression rates, alterations in *p53* correlate with more advanced disease. Kiyoshima et al. reported that all cases with *p53* mutations in their study showed either recurrence or metastasis [87]. Similarly, another study found that 57.1% of recurrent tumors had alterations in *p53*, compared to 14.3% of primary ACCs [86]. These findings suggest that mutations in *p53* may not be critical in the early tumorigenesis of ACC but may be important in later stages of tumor progression and recurrence. Furthermore, Yamamoto suggests that lower grade ACCs may progress to higher grade tumors, in part through mutations in *p53* [85, 89]. Overall, however, it seems that more studies are needed to truly determine the significance of *p53* on ACC pathogenesis.

p16 ^{INK4A}—*P16*^{INK4A} is a tumor suppressor gene localized to 9p21. It encodes a protein that inhibits cyclin-dependent kinases 4 and 6, which are in turn necessary for the phosphorylation of the retinoblastoma protein (*Rb*). Hypophosporylated *Rb* induces G1 arrest of the cell cycle [90–92]. Functional loss of *p16*, has been reported in many human neoplasms, including cancers of the head and neck [75, 93, 94].

Alterations in *p16*^{INK4A} are infrequent in ACC, occurring with a frequency of 14–20% of tumors [95, 96], and may be due to either genetic or epigenetic changes. Nishimine observed both homozygous deletion and promoter methylation of *p16*^{INK4A} but proposed that the latter may be particularly important in ACC tumorigenesis [96]. Functionally, loss of *p16*^{INK4A} has also been associated with more advanced disease and increased cell proliferation [95]. All in all, however, the results of these studies are rather preliminary and further research on this gene's role in ACC are still needed.

FHIT* and *WWOX*—FHIT*** and ***WWOX*** are tumor suppressor genes that encompass the ***FRA3B*** and ***FRA16D*** fragile sites at chromosomes 3p14.2 and 16q23.3, regions sensitive to chemical, environmental and viral damage. Both genes have been implicated in DNA damage response and the induction of proapoptotic signals [97]. Their loss has been associated with the development of many types of malignancies. Recently, Dincer et al. [98] showed using immunohistochemistry that ***FHIT*** and ***WWOX*** expression were significantly reduced in ACC but not in mucoepidermoid carcinoma. This difference could be attributed to the basaloid nature of ACC, whereas mucoepidermoid carcinoma is a glandular neoplasm. Similarly, in breast cancer, both ***FHIT*** and ***WWOX*** were significantly associated with basal-like phenotypes [99]. Taken together, these results suggest that either: (i) ***FHIT*** and ***WWOX*** are important for the progression in basaloid tumors such as ACC, or (ii) chromatin differences in basal cells allow these genes to be deleted, rearranged, or methylated early on in the neoplastic process.

Interestingly a promoter methylation study performed on several TSG's including ***FHIT*** showed that ***FHIT*** promoter methylation rates were actually much higher in normal tissue than in benign or malignant salivary gland tumors [100]. The reason for this inverse relationship is unclear.

b. Adhesion Molecules

Alterations in a variety of adhesion molecules have been shown to play a role in later stages of tumor progression, including cellular de-differentiation, local invasion, and metastatic spread. Loss of adhesion may allow neoplastic cells to separate from the primary tumor, migrate through the extracellular matrix, and metastasize to other sites, where new adhesive interactions are required for subsequent growth [101]. In these processes of tumor spread, changes in cell surface molecules, other modulators of cell adhesion, and basement membrane components may all be contributory. The role of adhesion molecules and related proteins is of particular interest in the progression of ACC, given that one hallmark of the tumor is its propensity for local invasion and late distant metastasis. In addition, reliable markers of local invasion and distant spread could offer prognostic value in the clinical setting.

Families of adhesion molecules that have been investigated in ACC include the cadherins, integrins, and the immunoglobulin superfamily of receptors.

Cadherins—_E-cadherin_, a transmembrane glycoprotein, is expressed on epithelial cells and mediates calcium-dependent cell-cell adhesion. Its extracellular domain mediates interactions between cells, while its cytoplasmic domain binds to β- or γ-*catenin* [102, 103]. We previously discussed the importance of the *Wnt/β-catenin* pathway in the pathogenesis of ACC. As it turns out, *E-cadherin* has been shown to keep this signaling pathway in check by sequestering β-*catenin* at the membrane, preventing it from translocating to the nucleus to transmit *Wnt*-induced signals [104]. *E-cadherin* levels essentially serve to set the threshold which must be overcome by *Wnt* to provoke β-*catenin* relocation and signaling. Thus, decreased *E-cadherin* levels can lead to overstimulation of the *Wnt/β-catenin*-induced cell proliferation and motility [105]. These effects have been demonstrated in various tumor types that show increased invasiveness and metastatic potential correlating to a down-regulation in *E-cadherin* expression [106, 107].

In ACC, loss of *E-cadherin* appears to play a role only in the later progression of the tumor. Franchi et al. found that *E-cadherin* expression varied inversely with tumor grade and stage, tumor size, extent of tumor infiltration, and presence of distant metastases. Of note, decreased *E-cadherin* expression was predictive of poorer clinical outcomes [108].

Functional loss of *E-cadherin* may be due to gene silencing via genetic or epigenetic alterations, or alternatively, to alterations in β-*catenin*. *E-cadherin* function depends on the integrity of the cadherin-catenin complex, so disruptions in β-*catenin* structure can prevent *E-cadherin* from binding and sequestering it at the membrane [101]. Interestingly, studies have shown that both β-*catenin* and *E-cadherin* expression decrease concomitantly in ACC [63, 109]. Furthermore, Daa et al. showed that with increasing histological grade, both β-*catenin* and *E-cadherin* became more sparsely and irregularly distributed. These findings suggest that changes in the expression and distribution of both β-*catenin* and *E-cadherin* may contribute to ACC pathogenesis [63].

Integrins: β-*6-integrin* is a subunit of an integrin heterodimer and is expressed solely in epithelium during tissue repair and tumorigenesis [110]. Westernoff found that expression of *beta-6 integrin* was higher in malignant salivary gland tumors, including ACCs, than in benign tumors. This suggests that β-*6-integrin* may play some role in promoting the invasiveness of malignant tumors [111].

Immunoglobulin superfamily of receptors: Adhesion molecules in the immunoglobulin receptor superfamily include *NCAM* (neural cell adhesion molecule), *HCAM* (homing cell adhesion molecule), *PECAM-1* (platelet-endothelial cell adhesion molecule), and *ICAM-1* (intercellular adhesion molecule). *NCAM* is expressed by peripheral nerve sheath cells and its expression has been reported in 31–100% of cases of ACC, [112–115]. However, no correlation was found between the intensity of staining and either perineural invasion or recurrence [112, 114]. *HCAM, PECAM-1*, and *ICAM-1* have also been studied; however, no correlation was found between level of expression and invasion or recurrence in any of these molecules. Expression patterns of *HCAM* and *ICAM-1* were similar to those of normal salivary gland tissue; alteration in the expression of these proteins does not appear to occur with malignant transformation. One case of ACC with a poor clinical outcome showed weak *PECAM-1* staining of tumor cells, but further studies are needed to elucidate the role of *PECAM-1* in ACC progression [112].

IV. DNA METHYLATION IN ACC

DNA methylation changes, including both hypomethylation and hypermethylation, are commonly found in human cancers and can result in aberrant activation of oncogenes and silencing of tumor suppressor genes without alterations in DNA sequence [116, 117]. Published reports on methylation in ACC mostly focus on candidate genes known to play important roles in non-ACC tumors [100, 118–123]. In some studies, differentially methylated genes were further correlated with available clinical and pathological parameters of the given tumor samples [121, 122], to identify their biological function and clinical importance (*see* Table 3).

Hypermethylation of Tumor Suppressor Genes

The majority of early methylation studies focused on a panel of well-characterized tumor suppressor genes identified in studies of other tumors. One of the first epigenetic studies published was done by Li et al. [121] who analyzed the frequencies of promoter methylation in four tumor suppressors genes: $p16^{INK4A}$, *RAS*-associated domain family protein 1A (*RASSF1A*), death-associated protein kinase (*DAPK*) and O^6-methylguanine-DNA methyltransferase (*MGMT*) in 60 ACC tumor samples. Using methylation-specific PCR (MSP), they found that $p16^{INK4A}$, *RASSF1A*, *DAPK* and *MGMT* exhibited promoter methylation in 47%, 42%, 16% and 7% of examined ACC samples, respectively. Forty-six tumors or 77% showed DNA methylation in one or more of the four aforementioned promoters. Li et al [121] also demonstrated that the promoter of *E-cadherin* was methylated

in 57% of their ACC tumor samples [122]. This frequency was lower than an earlier report of 70% *E-cadherin* methylation in ACC by Maruya et al [120], the difference possibly attributed to a smaller sample size in the latter analysis.

Furthermore, Li *et al* [121] also showed that promoter methylation of *RASSF1A* was significantly correlated with advanced tumor stage whereas promoter methylation of *E-cadherin* was significantly associated with perineural invasion. Moreover, both *E-cadherin* and *RASSF1A* methylation were significantly associated with high-grade ACC. Although this study was one of the first to show the importance of epigenetics in ACC, a major limitation of this study is that normal salivary gland tissue was not included in the analysis. Without a normal control for comparison, it is hard to define the standard level of methylation that occurs in salivary gland tissue versus that which is abnormal and ACC specific.

Williams et al [123] performed a similar analysis on 26 ACC and 29 normal salivary gland tissue samples using MSP. They reported promoter methylation of *DAPK*, *MGMT*, retinoid acid receptor β2 (*RARβ2*), and *RASSF1A* in 8%, 4%, 4% and 15% of tumor samples, respectively, whereas no methylation was detected in the normal tissue. In the concurrent analysis of these four genes, 30.8% of ACC samples showed promoter methylation in at least one gene. The differences in methylation rates found in Li's study versus this one might reflect differences in the anatomical sites from where the tissue was obtained, differences in tumor grade, and differences in PCR technique, thereby indicating the importance of standardization in these types of analyses.

Recently, promoter methylation was evaluated in a large panel of 19 well-known TSGs, in 17 ACC and 17 normal salivary gland tissues, using quantitative real-time MSP [100]. Among those TSGs, *APC*, *Mint1* and *RASSF1A* showed promoter methylation in 35.3%, 52.9% and 35.3% of ACC comparing to 0%, 17.6%, and 0% in normal samples, respectively. A very interesting finding was that quite a few TSGs showed high levels of methylation in ACC as well as normal salivary gland tissues. For example, *HIC1* and *14-3-3 σ* showed 94.1% and 100% of methylation in ACC, and 100% methylation of both genes in normal salivary specimens. This study suggested that it is crucial to utilize normal salivary gland tissues as controls in interpreting the meaning of promoter hypermethylation of TSGs.

14-3-3 σ is a gene of interest in ACC. Although the aforementioned study showed no difference in *14-3-3 σ* methylation between tumor and normal tissues[100], Uchida *et al*[118] suggest that epigenetic silencing of this gene may be important in ACC. *14-3-3 σ* negatively regulates the cell cycle by binding to cyclin/CDK complexes and inhibiting their interactions, thereby preventing cell cycle progression [124]. It has been shown to be required for stable G2 cell cycle arrest and also for activating *p53* after DNA damage [125]. *14-3-3 σ* is silenced via promoter methylation in many types of tumors [125]. In ACC Uchida *et al* [118] reported promoter methylation of *14-3-3 σ* in 8 out of 14 tumors and demonstrated that the aberrant methylation was in fact responsible for decreased gene expression. Furthermore, irradiation promoted heightened expression of *14-3-3 σ* and cell cycle arrest in normal cells but neither event occurred in ACC[118], suggesting that *14-3-3 σ* is responsible for proper DNA damage response and is abnormally silenced in ACC. The exact degree of epigenetic silencing of *14-3-3 σ* and its role in ACC needs to be better elucidated.

Cyclin-dependent kinase inhibitors (CKIs), much like *14-3-3 σ*, can bind to and coordinate the activities of cyclin/cyclin dependent kinases (CDKs) to regulate cell cycle progression [126]. However, unlike *14-3-3 σ*, they can be both positive and negative regulators. One particular member of the CKI family, *P27*, has been shown to be a prognostic marker for human cancers. Daa *et al* [125] analyzed promoter methylation of this

and other CKI genes including *p15*, *p18*, *p19*, and *p21* in 34 cases of ACCs by MSP. The incidences of promoter methylation in ACC tumors were 68.8% for *p15*, 90.3% for *p18*, 7.8% for *p19*, 92.3% for *p21*, and 26.5% for *p27*. They also showed that the expression of *p27* was diminished in tumor cells compared to that of surrounding normal tissue; the expression *of p15, p18, p19*, and *p21* were not examined in a comparison study.

The aforementioned studies identify promising candidate genes in ACC by demonstrating methylation in their promoter regions. However, complete data on gene expression and how they differ from that of normal salivary tissue is lacking for most genes studied. In fact, an important finding from the aforementioned Durr et al.'s [100] study is that although high methylation rates were found in several TSG loci, the methylation statuses of many of these genes were also high in normal tissue. The implication is that the comparison of methylation rates between normal and tumor tissue may be more informative than any high rate of methylation by itself. More comparison studies on ACC methylation are thus warranted.

Hypomethylation of Oncogenes

There is a great dearth of literature on aberrant methylation of oncogenes in ACC though hypomethylation of oncogenes is a widely accepted mechanism of oncogenesis. One recently published study looked at methylation of a majority of the *MYB* promoter to see if it played a role in the upregulation of *MYB* expression seen in ACC tumors [127]. However, bisulfite genomic sequencing of 18 primary ACC tumors and 13 normal salivary gland specimens demonstrated no detectable promoter methylation in any samples, suggesting that differential methylation is not a cause of *MYB* upregulation.

A reason for the relative lack of oncogene methylation literature in ACC is that genes chosen for study are often based what is known in other tumors and good candidate oncogenes have not yet been identified for methylation studies in ACC. However, it is quite plausible that there may be novel, yet unidentified genes playing a part in ACC pathogenesis. Therefore, a comprehensive screening for all genes differentially methylated in ACC may provide additional interesting targets Bell et al's study aimed to do this using a methylated CpG island amplification and microarray, followed by a pyrosequencing technique [128]. They found significant hypomethylation was identified in 7 CpG islands, specifically, located near *FBXO17, PHKG1, LOXL1, DOCK1*, and *PARVG* genes. Hypermethylation was found in 32 islands near genes encoding predominantly transcription factors, and 13 genes with various cellular functions such as stress response (*MT1H*), detoxification (*EPHX3*), protein catabolism (*AQPEP*), and apoptosis (*BCL2L11*), to name a few. Furthermore, they showed that *EN1*, a hypermethylated transcription factor, was correlated with histological grading of tumor, location of tumor, and final outcome of patient.

Bell et al.'s study demonstrates the likely direction that methylation analysis of tumors such as ACC will take in the future. A high-throughput comparison study of the methylation status of a large number of genes is useful in identifying novel targets for future investigation.

V. MITOCHONDRIAL MUTATIONS

The role of mitochondrial mutations in carcinogenesis is a relatively new and evolving topic. To date, only one study has performed an in-depth characterization of mitochondrial mutations in ACC. Mithani et al [129] used the MitoChip v2.0 (Affymetrix, Santa Clara, CA) resequencing array to sequence the mitochondrial genome of 22 ACC tumors and their matched leukocyte DNA. Of note, they found that 17 out of 22 ACC tumors carried mitochondrial mutations, most of which occurred in the NADH complex and a genome

region called the D-loop. The results of this study suggest that aerobic respiration changes may play a significant role in ACC carcinogenesis, but further research in this area is needed.

VI. BIOMARKERS OF DISEASE

All of the aforementioned described chromosomal and genetic changes in ACC lead to various phenotypic cellular changes that can be detected by various biomarkers. A few significant ones that have shown to be predictive of ACC disease progression and associated with prognosis include *ki-67* [130, 131], *p63* [132], and *TUNEL* [130].

The biomarker most frequently correlated with ACC prognosis is *ki-67*. *Ki-67* has been a widely studied marker of numerous malignancies and is commonly associated with cellular proliferation. Among salivary gland tumors, *ki-67* has been shown to have significantly higher expression in ACC than in polymorphous-low grade adenocarcinoma [133, 134] providing a useful tool to differentiate between the two tumor types. But most importantly, in ACC, high proliferation activity as measured by *ki-67* expression levels have been strongly correlated with decreases in patient survival rate [130, 131].

Similarly, high expression of *p63*, a widely studied marker of basal cells in normal salivary glands and of tumor cells from various malignancies, has also been correlated with decreased survival in ACC patients [132]. However, a direct role for *p63* in tumorigenesis has not yet been demonstrated.

Lastly, *TUNEL* is a commonly used assay for apoptosis rates. In ACC, high levels of *TUNEL* staining have been correlated with metastasis, extracapsular spread, grade and stage. Moreover, the 5-year survival rate was shown to drop in proportion to the *TUNEL* staining level [130].

Discovery of new and accurate biomarkers is of considerable importance. Currently, ACC follows an unpredictable course with an uncertain prognosis after surgical resection. If future research on biomarkers could identify patients with worse prognoses or higher risk of recurrence, this would allow for increased accuracy in patient counseling and treatment guidance.

VII. CONCLUSION

In summary, there has been marked progress in the exploration of the molecular basis of ACC development, but clearly there is much that remains unknown. Because of its relative lack of known etiologies, it is presumed that ACC arises spontaneously. Thus, one might assume that the molecular fingerprint is relatively simple. While this may be the case, the critical factors involved remain elusive. It remains our hope that through the basic research of ACC, discoveries will ensue that will impact the treatment and outcomes of patients with this unusual and progressive disease.

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Table 1

Chromosomal abnormalities in ACC detected via cytogenetic methods.

Cytogenetic Method	Technique	Resolution for detecting chromosomal gains/losses	Result
G-banding	Metaphase chromosomes treated with trypsin and stained with giemsa	Poor	6q22-6q2S deletion[2,3]
Fluorescent in situ Hybridization (FISH)	Fluorescent probes that bind to regions of DNA with sequence similarity	Moderate	t(6;9)(q21–24; p13–23)[6;] fusion *MYB-NFIB*[8,10]
Comparative Genomic Hybridization (CGH) and array-based CGH	Tumor and normal DNA fluorescently labeled, mixed and hybridized to metaphase chromosomes or slide with DNA probes (in array-based CGH). Regional differences in fluorescence indicate gains/losses	Moderate-high	loss at: 12q12-q13[12,15], 1p32–36[13] gain at: 22q12-q13[11,14,15], 18[13], 16p[11,15] 17q[11,15], 18[13] t6:9[15]
Microsatellite Marker and Loss of Heterozygosity Analysis	Tumor and normal DNA digested and ligated to adaptors and amplified. Amplified DNA hybridized to gene chip and copy number determined by subsequent hybridization intensity of SNP probe.[20]	High	loss at: 12q13.11-q13.3[19], 12q24.32-q24.33[19], 6q24.1-q25.1[20]

Table 2

Oncogenes involved in ACC pathogenesis

Type	Protein	Normal Function	Role in ACC
Developmental proteins	***Sox4***	Proper development of the cardiac outflow tract, pro-B-lymphocyte differentiation, and CNS development[23, 24]	Cell survival, proliferation, and apoptosis[25] Possibly plays role in metastasis[27]
	c-kit	Binds stem cell factor and promotes cell growth and differentiation[29] Hematopoiesis, spermatogenesis, and growth and migration of melanocytes[30,31]	Expression correlates with tumor grade[32] Acts with *Slug* to control cell migration[42] Associated with advanced stage, perineural invasion, local regional recurrence and metastases[40]
	MYB	Various; cell cycle regulation	Fusion *MYB-NFIB* transcripts causes *MYB* overexpression[9,10,11]
Growth factors	***EGF/ EGFR***	Various; cell proliferation and survival	Cell survival, proliferation, oncogenesis, and metastasis48,49,50, *EGFR* expression correlates with tumor grade but not patient prognosis[52]
	VEGF/ VEGFR	Angiogenesis[53,54]	Increases microvessel density[56,57] Prognosticator of survival[56,58]
	BDNF and NGF	Neutrophins; promote survival, differentiation, and function of neurons[44].	Facilitating perineural invasion[44,61]
Signaling molecules	***Wnt/ 6-catenin***	Activates genes important for growth and proliferation[65,66]	Reduced membranous expression of *6-cotentn* associated with metastasis[67] Expression of *Galectin-3*, a regulator of *Wnt*/β-catenin[69], is associated with metastases and a more aggressive tumor phenotype[69,72]

Table 3

Frequency of *MYB-NFIB*- translocation in ACC tumors

Study	Number of ACC Tumors	Translocation and Frequency	Notes
Persson et al.[9]	12 head and neck ACC tumors	*MYB-NFIB* detected in all ACC tumors and none of non-ACC tumors	
Mitani et al.[10]	123 primary and metastatic ACC tumors	*MYB-NFIB* present in 28% of primary tumors and 35% of metastatic. Not present in other tumor types	*MYB* was overexpressed in the majority of ACC tumors and significantly higher expression was found in the fusion transcripts.
West et al.[11]	37 salivary ACC tumors	*MYB-NFIB* present in 49% of ACC's. Not present in other tumor types	*MYB* immunostaining is confined to the basal cell component though the translocation is present in all the cells

Table 4

Tumor Suppressor Genes (TSGs) involved in ACC pathogenesis

Type	Protein	TSG Function	Role in ACC	Method of Inactivation
Cell cycle regulators	*p53*	Causes G1 arrest via *p21* and triggers apoptosis [76,77,78]	Involved in recurrence or metastasis[86,87] Progression to more advanced disease[85,89]	Missense mutation[79–81]
	p16^{INK4A}	G1 arrest of cell cycle via phosphorylation of *Rb*	Associated with more advanced disease and increased cell proliferation[95]	Homozygous deletion[95,96] Methylation[96]
	FHIT and WWOX	DNA damage response and induction of proapoptotic signals[97]	Associated with progression of basaloid tumors[98,99]	Chromosomal loss[97] Methylation[100]
	14-3-3 δ	Prevents cell cycle progression by binding cyclin/CDK complexes and inhibiting their interaction[124] Activating ρ*S3* after DNA damage[125]	Expression decreased in ACC[118] Irradiation did not promote heightened expression in ACC but did in normal samples[118]	Methylation[118]
	RASSF1A	Interacts with a number of signaling molecules involved in cell growth, survival, and apoptosis	Promoter methylation correlated with advanced tumor grade and stage[117]	Methylation96,117,119
Adhesion molecules	*E-cadherin*	Expressed on epithelial cells and mediates calcium-dependent cell-cell adhesion[102,103] Sequesters 6-*catenin* at membrane[104]	Expression correlates inversely with tumorgrade and stage[108] Promoter methylation associated with perineural invasion[121]	Mutations[106–108] Methylation[121,122] Change in *6 catenin* structure[97]
	Beta-6 Integrin	Tissue repair[110]	Invasion[111]	Unknown
	NCAM Ineural cell adhesion molecule)	Immunoglobulin expressed by peripheral nerve sheath cells[112–115]	Overexpressed but no correlation was found with perineural invasion or recurrence[112,115]	Unknown