Pathogenesis of NUT Midline Carcinoma

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Abstract

NUT midline carcinoma (NMC), an aggressive form of squamous cell carcinoma, is defined by the presence of acquired chromosomal rearrangements involving NUT, usually BRD4-NUT fusion genes and, less commonly, NUT-variant fusion genes involving BRD3 or still-uncharacterized genes. Improved diagnostic tests reveal that although rare, NMCs occur in people of any age and may be indistinguishable from more common squamous cell carcinomas of adulthood. NMCs have simple karyotypes whose hallmark is genomic instability, suggesting that NMC arises through a distinct pathogenic pathway representing a genetic shortcut to the phenotype of squamous cell carcinoma. Mechanistically, BRD-NUT fusion proteins appear to act by blocking differentiation, possibly by sequestering histone acetyltransferase activity. Accordingly, histone deacetylase inhibitors or BET inhibitors, the latter of which inhibit binding of BRD-NUT proteins to chromatin, induce terminal differentiation of NMC cells. These insights provide a rationale for targeted therapy of NMC, which is almost uniformly refractory to conventional chemotherapy and radiotherapy.

Keywords

BRD4-NUT, BET inhibitor, histone deacetylase inhibitor, histone acetyl-transferase, http://www.NMCRregistry.org, differentiation therapy
NMC: NUT midline carcinoma
Carcinoma: malignant neoplasm of epithelial cells
Chromosomal rearrangement: structural aberration of a chromosome; includes deletions, duplications, inversions, and translocations
Ewing sarcoma: a rare, malignant, round-cell tumor arising within bone or in soft tissue
Squamous: literally, flat or scale-like; describes, for instance, the epidermis of skin, which is composed of squamous epithelium
Epigenetic: refers to heritable alterations in gene expression caused by mechanisms other than DNA sequence changes; histone acetylation and methylation are well-known examples

CLINICAL FEATURES OF NUT MIDLINE CARCINOMA

NUT midline carcinoma (NMC) is a rare, aggressive cancer. It can affect any gender or age group (Table 1) and occurs equally in males and females from the neonatal period (1) through the eighth decade of life (2). The cell of origin is not known: No in situ epithelial component has ever been observed, and the tumor is usually locally invasive and widely metastatic at diagnosis. Because NMCs arise from various anatomical sites (Table 1), they cannot be categorized by the same system of nomenclature as most other carcinomas, which are traditionally defined by the tissue of origin. Instead, NMC is defined genetically by the presence of chromosomal rearrangements involving the NUT gene (3). The term midline is applied because of NMC’s tendency to arise from midline anatomical sites, most commonly the upper aerodigestive tract (50%) and the mediastinum (41%) (Table 1) (4). However, it has been diagnosed within such varied tissues as the parotid gland (5,6), pancreas (1), adrenal gland (M. O’Sullivan & C.A. French, unpublished data), subcutis (C.A. French & E. Salman, unpublished data), bladder (3), and iliac bone (7).

Apart from these unique features, NMC is best known for its devastating clinical course. Of the 62 cases known to this author (C.A. French, unpublished observations), only one patient has been cured (7). NMC is often initially responsive to chemotherapy and radiation, but it invariably recurs rapidly and does not respond to subsequent therapeutic interventions. The average survival is only 9.5 months (4), despite its frequent occurrence in previously healthy children or young adults without comorbid conditions. The one cured case was in many ways atypical. It arose within the iliac bone of a 10-year-old boy and was pathologically and clinically indistinguishable from Ewing sarcoma, except that it had the BRD4-NUT translocation and lacked EWS rearrangement. It is possible, therefore, that this singular case was a Ewing sarcoma associated with BRD4-NUT rearrangement. In line with this possibility, several subsequent typical NMCs were treated with Ewing sarcoma regimens, but they did not show any improved therapeutic response (8).

CYTOGENETICS

A unique feature of NMC is its cyogenetics. Most carcinomas, including typical squamous cell carcinomas of adulthood, have very complex karyotypes and are aneuploid (classically, a reciprocal t(15;19)(q14;p13.1)) and few, if any, other cytogenetic abnormalities (Figure 1). The cytogenetic features of NMCs and their occurrence even in very young children suggest that, as is the case for translocation-associated hematopoietic cancers, the genesis of NMC may involve relatively few mutations. This finding stands in contrast to mutagen-associated squamous cell carcinomas of adulthood, which arise over a period of many years and are associated with the accumulation of thousands to tens of thousands of mutations (10).

The basis for this genetic distinction is unclear. In hematopoietic tumors, the cell of origin is often a stem-like cell with the capacity to circulate widely between tissues and the blood (11); thus, spread from tissue to tissue is an ingrained property of most hematopoietic tumors. In contrast, acquisition of the ability of carcinomas to invade tissue and metastasize may require a complex series of genetic and epigenetic changes (12, 13), which may be driven by a high level of genomic instability. NMC may bypass the requirement for genomic instability due to origination from a stem-like cell with migratory properties or because NUT fusion proteins modify the epigenome of the cell of origin such that it acquires these properties.

MOLECULAR GENETICS

Approximately two-thirds of NMCs have a reciprocal chromosomal translocation involving NUT (nuclear protein in testis, cbr15orf35) on...
### Table 1  Clinical characteristics of reported NUT midline carcinomas

<table>
<thead>
<tr>
<th>Case number</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Location</th>
<th>Diagnosis</th>
<th>Survival (months)</th>
<th>BRD4-NUT</th>
<th>NUT variant</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>M</td>
<td>Bladder</td>
<td>PD squamous cell carcinoma</td>
<td>8.5</td>
<td>Present</td>
<td>Absent</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>M</td>
<td>Mediastinum</td>
<td>Mucoepidermoid carcinoma</td>
<td>3.5</td>
<td>Present</td>
<td>Absent</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>M</td>
<td>Iliac bone</td>
<td>Ewing sarcoma/ PNET AWOD</td>
<td>4.5</td>
<td>Present</td>
<td>Absent</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>F</td>
<td>Thorax</td>
<td>Undifferentiated carcinoma</td>
<td>3.25</td>
<td>Present</td>
<td>Absent</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>F</td>
<td>Nasopharynx</td>
<td>PD squamous cell carcinoma</td>
<td>9</td>
<td>Present</td>
<td>Absent</td>
<td>23</td>
</tr>
<tr>
<td>6</td>
<td>13</td>
<td>F</td>
<td>Epiglottis</td>
<td>Undifferentiated carcinoma</td>
<td>6</td>
<td>Present</td>
<td>Absent</td>
<td>24</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>M</td>
<td>Mediastinum</td>
<td>Thymic PD squamous cell carcinoma</td>
<td>7.5</td>
<td>Present</td>
<td>Absent</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>15</td>
<td>F</td>
<td>Orbit</td>
<td>PD squamous cell carcinoma</td>
<td>7</td>
<td>Present</td>
<td>Absent</td>
<td>33, 16</td>
</tr>
<tr>
<td>9</td>
<td>16</td>
<td>M</td>
<td>Lung</td>
<td>Squamous cell carcinoma</td>
<td>37</td>
<td>Absent</td>
<td>Present</td>
<td>3, 16</td>
</tr>
<tr>
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<td>16</td>
<td>F</td>
<td>Trachea</td>
<td>PD carcinoma</td>
<td>31.5</td>
<td>Absent</td>
<td>Present</td>
<td>33, 16</td>
</tr>
<tr>
<td>11</td>
<td>21</td>
<td>F</td>
<td>Nasopharynx</td>
<td>Nasopharyngeal carcinoma</td>
<td>10.25</td>
<td>Absent</td>
<td>Present</td>
<td>3</td>
</tr>
<tr>
<td>12</td>
<td>22</td>
<td>F</td>
<td>Thymus</td>
<td>PD carcinoma</td>
<td>3.5</td>
<td>Present</td>
<td>Absent</td>
<td>20</td>
</tr>
<tr>
<td>13</td>
<td>26</td>
<td>M</td>
<td>Sinonasal</td>
<td>Undifferentiated carcinoma</td>
<td>16.75</td>
<td>Present</td>
<td>Absent</td>
<td>3</td>
</tr>
<tr>
<td>14</td>
<td>30</td>
<td>F</td>
<td>Mediastinum</td>
<td>PD carcinoma</td>
<td>3.25</td>
<td>Present</td>
<td>Absent</td>
<td>8</td>
</tr>
<tr>
<td>15</td>
<td>31</td>
<td>M</td>
<td>Nasal cavity</td>
<td>Sinonasal undifferentiated carcinoma</td>
<td>—</td>
<td>Present</td>
<td>Absent</td>
<td>2</td>
</tr>
<tr>
<td>16</td>
<td>34</td>
<td>F</td>
<td>Thorax</td>
<td>NK</td>
<td>NK</td>
<td>Present</td>
<td>Absent</td>
<td>Dang et al. 2000</td>
</tr>
<tr>
<td>17</td>
<td>34</td>
<td>M</td>
<td>Mediastinum</td>
<td>PD carcinoma</td>
<td>2</td>
<td>Absent</td>
<td>Present</td>
<td>16</td>
</tr>
<tr>
<td>18</td>
<td>35</td>
<td>F</td>
<td>Mediastinum</td>
<td>PD carcinoma</td>
<td>2</td>
<td>Absent</td>
<td>Present</td>
<td>3</td>
</tr>
<tr>
<td>19</td>
<td>39</td>
<td>F</td>
<td>Nasal cavity and sinus</td>
<td>PD squamous cell carcinoma</td>
<td>—</td>
<td>Present</td>
<td>Absent</td>
<td>2</td>
</tr>
<tr>
<td>20</td>
<td>40</td>
<td>F</td>
<td>Nasal cavity and sinus</td>
<td>PD squamous cell carcinoma</td>
<td>—</td>
<td>Present</td>
<td>Absent</td>
<td>2</td>
</tr>
<tr>
<td>21</td>
<td>47</td>
<td>M</td>
<td>Nasal cavity and sinus</td>
<td>Sinonasal undifferentiated carcinoma</td>
<td>—</td>
<td>Present</td>
<td>Absent</td>
<td>2</td>
</tr>
<tr>
<td>22</td>
<td>78</td>
<td>F</td>
<td>Supraglottic larynx</td>
<td>Undifferentiated carcinoma</td>
<td>—</td>
<td>Present</td>
<td>Absent</td>
<td>2</td>
</tr>
<tr>
<td>Average</td>
<td>25.14</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>9.53</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

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Not included in average survival.

These variants are BRD3-NUT. Abbreviations: AWOD, alive without disease; PD, poorly differentiated; NK, not known; PNET, primitive neuroectodermal tumor.
Figure 1

(Left) Karyotypes of typical non–NUT midline carcinoma (NMC) squamous cell carcinoma, compared with (right) NMC. The red arrows denote chromosomal translocations between chromosomes 15 and 19. Left panel adapted with permission from Dr. Susan Gollin and Elsevier, Ltd. (9).

**Bromodomain and extraterminal domain–containing protein (BET protein):** binds acetylated lysine residues; often termed a reader

**Oncogene:** a gene that can cause cancer

**Histone acetyltransferase (HAT):** an enzyme, often termed a writer, that acetylates histone residues, most commonly lysine

**Differentiation:** a maturation process whereby a less specialized cell becomes a more specialized cell

chromosome 15q14 and the BET family gene BRD4 (also known as MCAP and HUNK1) on chromosome 19p13.1, which forms an in-frame BRD4-NUT fusion oncogene whose product is driven by the BRD4 promoter (Figures 1 and 2). The BRD4-NUT fusion gene contains the first half of BRD4, which includes all of its known functional domains: two paired bromodomains, an extraterminal (ET) domain, and a bipartite nuclear localization signal (NLS). The NUT portion of the fusion gene contains essentially the entire coding region for NUT, a largely unstructured polypeptide highly expressed in normal spermatocytes that has an acidic binding domain for the histone acetyltransferase (HAT) p300 (14). Although the t(15;19) is reciprocal, the other product of the translocation, NUT-BRD4, is not expressed, in accordance with the testis-restricted activity of the NUT promoter (15).

Less commonly, NMC harbors a different rearrangement involving NUT (Figure 2) (3). A subset (approximately 25%) of these cases have BRD3-NUT rearrangements (16). The BRD3-NUT fusion gene also drives the expression of a chimeric protein consisting of the two bromodomains, an ET domain, and NLS of BRD3, fused to the entirety of NUT (Figure 2). The remaining genes involved in other NUT-variant rearrangements are unidentified; it will be of interest to determine whether these genes encode proteins that serve similar functions or whether they are part of the same functional complex as BRD4 and BRD3 (described below). On the basis of outcomes in a small number of cases, it has been suggested (3) that NUT-variant NMCs may be associated with longer survival.

**HISTOPATHOLOGY**

The histopathology of NMC is characteristic, but not diagnostic. The most common appearance is that of a poorly differentiated carcinoma with focal, abrupt squamous differentiation (Figure 3). In contrast to many other poorly differentiated carcinomas, which consist of highly pleomorphic large cells (Figure 3), NMC cells are usually medium sized, round, and often monomorphic in appearance. Overt areas of squamous differentiation are seen in approximately half of cases (3; C.A. French,
unpublished observations) but may not always be present, particularly in small biopsies. The histopathologic features of NMC overlap with those of several other poorly differentiated cancers (Table 2), including poorly differentiated squamous cell carcinoma, sinonasal undifferentiated carcinoma (2), Ewing sarcoma, Epstein-Barr virus–associated nasopharyngeal...
Table 2  Initial diagnoses in cases of NUT midline carcinomas

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous cell carcinoma</td>
<td>12</td>
</tr>
<tr>
<td>Poorly differentiated carcinoma</td>
<td>8</td>
</tr>
<tr>
<td>Sinonasal undifferentiated carcinoma</td>
<td>3</td>
</tr>
<tr>
<td>Malignant neoplasm, not otherwise specified</td>
<td>2</td>
</tr>
<tr>
<td>Thymic carcinoma</td>
<td>2</td>
</tr>
<tr>
<td>Ewing sarcoma</td>
<td>2</td>
</tr>
<tr>
<td>Leukemia</td>
<td>2</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>1</td>
</tr>
<tr>
<td>Pancreatoblastoma</td>
<td>1</td>
</tr>
<tr>
<td>Carcinoma expleomorphic adenoma</td>
<td>1</td>
</tr>
<tr>
<td>Nasopharyngeal carcinoma</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>35</strong></td>
</tr>
</tbody>
</table>

carcinoma, thymic carcinoma, neuroblastoma and pancreatoblastoma (1), and even primary salivary gland carcinoma (5).

NMC is commonly misdiagnosed; it has even been mistaken for acute leukemia (due to the occasional expression of CD34) (Table 2). Also contributing to the failure to diagnose NMC are its rarity, physicians' failure to consider it in adult patients, and until recently, the absence of widely available diagnostic tests (described in the following section). To counter the lack of awareness of NMC among pathologists and oncologists, an Internet-based international NMC registry (http://www.NMCRRegistry.org) has been established. This registry provides access to (a) pathologic review, (b) updated information about the disease, (c) treatment guideline suggestions, (d) a repository for clinical data, and (e) educational information for physicians and patients about this disease.

DIAGNOSIS

The diagnosis of NMC depends on the demonstration of NUT gene rearrangement or misexpression. Although cytogenetic demonstration of a t(15;19) by karyotyping is sufficient for a presumptive diagnosis of NMC (Figure 1), most diagnoses are based on formalin-fixed, paraffin-embedded (FFPE) tissues because few suspected carcinomas are submitted for karyotyping. To fill this diagnostic need, we developed a fluorescence in situ hybridization assay that uses dual-color, break-apart bacterial artificial chromosome probes flanking the NUT and BRD4 loci (Figure 4a) (15, 17, 18). This assay can be used on virtually any specimen preparation (except for those with heavy-metal fixation), including frozen tissue, acetic acid–fixed cytogenetic preparations, thin (4–5-μm) sections of FFPE tissues or disaggregated cells, and air-dried or ethanol-fixed slides. Because this approach works on archival, formalin-fixed tissue, retrospective analysis can be performed on samples that are decades old (3).

If fresh or frozen tissue is available, a faster approach, reverse-transcriptase polymerase chain reaction, can be employed by use of primers flanking the known BRD4 and NUT break points (Figure 4b) (8). However, this method overlooks NUT-variant fusion genes.

To develop a diagnostic test for NMC that can be used routinely in the community, we raised and characterized a monoclonal antibody to NUT (clone C52) that detects NUT expression by immunohistochemistry (19). In a study that included a large number of control tissues, such as other forms of carcinoma, nuclear reactivity with this antibody was 100% specific and 87% sensitive for the diagnosis of NMC. Interestingly, the nuclear NUT staining appeared speckled in most cases (Figure 4c), which may have implications for the function of BRD-NUT, as discussed further below.

DEMographics and PREvalence

Improvements in diagnosis have led to increased recognition of NMC, but its true prevalence remains unknown. Notably, the antibody study described above (19) revealed that virtually none of the control tumors screened on tissue microarrays (TMAs) (N = 674) were discovered to be NMCs. However, most
Figure 4
Various molecular methods used to diagnose NUT midline carcinoma (NMC). (a) Fluorescence in situ hybridization using dual color probes flanking the NUT gene. The split-apart red and green signals indicate rearrangement of the NUT locus. Reproduced with permission from Reference 4. (b) Reverse-transcriptase polymerase chain reaction (RT-PCR) using primers that flank the coding sequence of the BRD4-NUT break point and appropriate controls. Reproduced courtesy of Yukichi Tanaka, Mio Tanaka, Toru Horisawa, and Yutaka Saikawa of Kanazawa Medical University, Ishikawa, Japan. (c) Immunohistochemistry on formalin-fixed, paraffin-embedded sections of carcinoma with the anti-NUT antibody. Produced by Cell Signaling Technologies, Inc., Danvers, Massachusetts. Reproduced with permission from Reference 19.

tumors on TMAs are at least partially resectable, whereas most NMCs are unresectable at presentation; thus, TMAs may be biased against inclusion of NMCs. Other screens have been intentionally biased toward tumor groups that are suspected of being enriched for NMCs. For example, screening of 98 visceral carcinomas in young patients (average age, 32.5 years) identified seven NMCs (7%) (3), and a second study that focused only on an uncommon group of undifferentiated carcinomas of the upper aerodigestive tract (N = 28) identified five NMCs (18%) (2). Interestingly, four of those five patients were over the age of 40 years, and one patient was a 78-year-old who carried a diagnosis of laryngeal squamous cell carcinoma.

The description of NMC in older patients (2) and the development of an
immunohistochemical test (19) have been associated with an increase in the number of NMC cases diagnosed. This increase, in turn, has provided a more complete picture of the demographics of NMC. For example, the first descriptions of this disease in children (3, 15, 17, 20–23) led to the perception that NMC was a pediatric cancer that affected mostly people in their first or second decade of life. In the cohort of patients that is known to us, there remains a pediatric-weighted age distribution (Figure 5a), but analysis of cases before 2008 (Figure 5b) versus those from 2008 on (Figure 5c) suggests that the prevalence of NMC is more evenly distributed among patients of all ages.

Given the age distribution of NMC, it is unlikely that smoking plays a pathogenic role; this hypothesis is supported by the absence of a smoking history in most, but not all, NMC patients (C.A. French, unpublished observations). None of the cases tested to date have been associated with Epstein-Barr virus or with human papilloma virus infection (2, 5, 17, 23; C.A. French, unpublished observations). NMC has been encountered in many parts of the developed world (Figure 5d) but is undoubtedly underrecognized in China and many other developing countries.

**BET PROTEINS**

The critical initiating oncogenic event in NMC is hypothesized to be the formation of NUT fusion oncogenes, most commonly BRD4-NUT. BRD4 is a ubiquitously expressed member of the family of double-bromodomain-containing, ET domain–containing BET proteins. BRD4 encodes two major isoforms. The short isoform is nearly entirely included in BRD4-NUT, whereas the long isoform encodes a long C-terminal domain (Figure 1) that is not present in BRD4-NUT and is implicated in the binding of papilloma viruses to host cell genomes (25). The paired bromodomains of BRD3/BRD4, both of which are present in BRD-NUT fusion proteins (Figure 2), bind specifically to acetylated histones associated...
with DNA (26–28). BRD4, originally termed mitotic chromosome-associated protein (29), binds acetylated chromatin throughout all phases of the cell cycle, including mitosis. This property is one basis for the hypothesis that BRD4 confers a sort of epigenetic memory to postmitotic daughter cells (29). The ability of BRD4 to facilitate transcriptional elongation of early postmitotic genes, such as cyclin D1, through its interaction with the positive transcriptional elongation factor P-TEFb supports this idea (30–32). In the context of NMC, the bromodomains of BRD3 and BRD4 probably play an important role in tethering NUT to chromatin (16). Notably, normal NUT shuttles between the cytoplasm and the nucleus in a CRM1-dependent fashion due to the presence of a nuclear export signal (Figure 2) (16), whereas BRD-NUT fusion proteins do not shuttle, instead remaining associated with chromatin throughout the cell cycle.

The ET domain is also present in BRD-NUT fusion proteins. It is structured (33) and binds the Kaposi sarcoma virus LANA-1 antigen (34), suggesting that it functions as a protein-protein interaction module.

**NUT PROTEIN**

NUT is a mammal-specific protein that is only moderately conserved. Human NUT has 66% amino acid identity with mouse NUT, compared with the 96% identity between human and mouse BRD4. NUT expression is normally restricted to postmeiotic spermatids. It is predicted to be a largely unstructured polypeptide, but contains two acidic, potential protein-binding domains (ADs) (Figure 2). One of these domains, AD1, is contained within a region that binds and activates p300 (35). On the basis of this association, Reynoird et al. (14) suggested that within spermatids, NUT binds and activates the HAT activity of p300, thereby leading to a global increase in histone acetylation, an epigenetic change that precedes the removal of histones and subsequent chromatin compaction during spermatogenesis. When fused to BRD, NUT appears to retain the capacity to bind p300, but it may do so in a way that impairs the expression of genes that are needed to drive the terminal differentiation of squamous epithelial cells (14, 16, 18). This idea is discussed in more detail below.

**BRD-NUT FUNCTION**

BRD-NUT proteins are toxic when expressed in heterologous cell lines (36; C.A. French, unpublished observations), and standard colony-forming assays (e.g., in NIH 3T3 cells) are not useful for the study of its transforming activities. However, long-term cultures of NMC cells obtained from patients are readily established, thereby providing a substrate for studies of BRD-NUT function. The striking observation is that knockdown of BRD4-NUT or BRD3-NUT induces rapid terminal squamous differentiation accompanied by arrested proliferation (Figure 6) (16, 18). Following knockdown, NMC cells begin to accumulate proteins associated with squamous differentiation, such as cytoplasmic keratin and involucrin, and demonstrate an increase in nuclear size and nuclear euchromatin. With additional time, these cells arrest their growth and terminally differentiate into flat, resting squamous cells (Figure 6). These responses to BRD-NUT withdrawal suggest that one of the major pathogenic consequences of BRD-NUT fusion proteins is to block differentiation, highlighting the therapeutic potential of targeting these proteins.

Given the known association of BRD4-NUT with acetylated chromatin and p300, a logical question was whether its expression alters histone acetylation globally. The answer is yes, but paradoxically, rather than increasing histone acetylation, BRD-NUT decreases it, a change that is associated with decreased expression of many genes (18). These findings suggested a model wherein BRD-NUT sequesters HAT activity or other factors that are required for expression of genes needed for terminal squamous differentiation. The sequestration model is supported by the observation that BRD-NUT localizes in a
bromodomain-dependent fashion to discrete nuclear foci (16) that are enriched for p300 and acetylated histones (Figure 7a) (14), yet transcriptionally inactive (Figure 7b) (14). Whether BRD-NUT interferes with transcription by physically sequestering HAT activity from differentiation-specific genes or through more subtle mechanisms remains to be clarified. Note that there is a testis-specific BRD family member, BRDT, that has been implicated in chromatin compaction in spermatids, which suggests that BRD-NUT fusion proteins may in some way mimic the normal activities of BRDT and NUT in the testis (26, 37, 38).

Assuming that the major effect of BRD4-NUT proteins is on differentiation, it follows that these oncoproteins are likely to collaborate with other, currently unknown mutations that increase growth and survival. The best-characterized and best-validated examples of this type of “oncogene conspiracy” are various combinations of transcription factor mutations and activating tyrosine kinase mutations in acute leukemia (39–41). With the advent of affordable deep sequencing (42), it is anticipated that the identification of collaborating mutations that are required for the growth of NMC cells will soon become possible. Such studies will rigorously assess the relative genomic stability of NMCs (as compared with other squamous cell carcinomas) and may lead to additional therapeutic opportunities.

PATHOGENIC INSIGHTS FROM SMALL-MOLECULE INHIBITORS

A nonneoplastic process in which HAT sequestration appears to play a role is Huntington disease, wherein expanded polyglutamine repeats sequester CBP/p300, resulting in global histone hypoacetylation and transcriptional repression (43–46). In Huntington disease, transcription can be restored by treatment with histone deacetylase inhibitors (HDACi) (44, 46), which restore the overall balance between HAT and HDAC activity. On the basis of the sequestration model for BRD-NUT proteins, we hypothesized that treatment with HDACi may tip the balance in favor of HAT activity outside of BRD-NUT foci, thereby restoring acetylation and transcription globally (Figure 8). In an initial test of this idea, we noted that treatment of cells transiently expressing green fluorescent protein (GFP)-BRD4-NUT with HDACi resulted in rapid dispersion of GFP-BRD4-NUT foci.
Immunofluorescence localization of BRD4-NUT and other proteins. (a) BRD4-NUT colocalizes with a number of acetylation marks associated with transcriptionally active DNA, including histone 3 acetyl lysine 9 (H3K9ac), acetyl lysine 14 (H3K14ac), acetyl lysine 5 (H3K5ac), and acetyl lysine 12 (H3K12ac). (b) BRD4-NUT localization relative to RNA polymerase II (RNApolII), and its active marks: C-terminal domain phosphoserine 2 (CTD S2Ph) and C-terminal domain phosphoserine 5 (CTD S5Ph). Scale bars, 10 μm.

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(Supplemental Video 1; follow the Supplemental Material link from the Annual Reviews home page at http://www.annualreviews.org). We then evaluated whether redistributing BRD-NUT proteins with HDACi could overcome the differentiation block that is characteristic of NMC cells. Indeed, we found that unlike non-NMC squamous cell carcinoma lines, treatment of NMC cells with low concentrations (25 nM) of the tool HDACi trichostatin A restored acetylation and led to the rapid differentiation and growth arrest of NMC cells (Figure 9a). The transcriptional profile and expression of select, differentiation-specific genes induced by HDACi closely mimicked those induced by small interfering RNA knockdown of BRD-NUT (18), suggesting that HDACi interferes with BRD-NUT function and reverses its blockade on differentiation.

With an eye toward potential therapy for NMC with HDACi, a panel of structurally unrelated HDACi was screened on multiple NMC cell lines; all were found to all be potently antiproliferative. Importantly, the antiproliferative activity of these HDACi on NMC cells closely correlated with increases in nuclear acetylation ($R^2 = 0.96$), which suggests that the inhibitory effects of the HDACi were on-target (18). Studies were then undertaken in three murine xenograft models of NMC. In all of these models, the investigational HDACi LBH-589 significantly inhibited growth and
Figure 8
Proposed model of how BRD-NUT may repress transcription and block differentiation by sequestering histone acetyltransferases (HATs) and cofactors (Co-Fs). Abbreviation: HDAC, histone deacetylase.

Suberoylanilide hydroxamic acid (SAHA): a histone deacetylase inhibitor also known as vorinostat or Zolinza®

induced differentiation (18) and, in extended studies, improved survival (Figure 9b).

During these investigations, a 10-year-old male with mediastinal BRD4-NUT NMC invading the left atrium and pulmonary vein was transferred to Children’s Hospital Boston. Because NMC is refractory to conventional therapies, treatment of this patient with SAHA (vorinostat), the only clinically approved HDACi used to date in children, was considered. Culture of patient-derived NMC cells confirmed that vorinostat induced squamous differentiation and growth arrest, and vorinostat given to the patient as a single
Figure 9
Effect of histone deacetylase inhibition on NUT midline carcinoma (NMC) in vitro and in vivo. (a) Morphologic and immunophenotypic changes consistent with differentiation (stained by hematoxylin and eosin and antikeratin AE1/AE3, respectively) and growth arrest (Ki-67 fraction) following trichostatin A (72 h, 25 nM) treatment of the NMC cell line TC-797 and the non-NMC squamous cell carcinoma cell line HTB-43. Magnification (scale bar, 25 μm) is identical for all panels. (b) (Top) Growth inhibition of xenograft model of NMC (PER-403) by the histone deacetylase inhibitor LBH-589. (Bottom) Survival analysis of PER-403 xenograft (n = 5 per group) treated with LBH589. (c) P18PF-fluorodeoxyglucose–positron emission tomography and computed tomography scan of the patient’s mediastinal tumor (arrow). Reproduced with permission from Reference 18. Abbreviation: ROI, roipoly polygonal region of interest.

agent for five weeks produced an objective response, as assessed by positron emission tomography (Figure 9c) (18). Unfortunately, SAHA therapy was complicated by marked thrombocytopenia, a well-known dose-limiting toxicity (47), and severe nausea and emesis, which became intolerable. Withdrawal of SAHA was followed by rapid recurrence of tumor growth, which was refractory to combination chemotherapy and led to the patient’s death 11 months after the initial diagnosis.

The glimmer of hope provided by the initial encouraging preclinical and clinical response to HDACi suggests that NMC may be amenable to therapy designed to induce differentiation. The best example of successful differentiation therapy at present is in the treatment of acute promyelocytic leukemia (APL) with all-trans retinoic acid (48–50), which overcomes a block in differentiation induced by the PML–RARα fusion oncprotein. However, further work is needed to understand in detail how BRD-NUT blocks differentiation and how HDACi overcomes this blockade.

A second lesson from the experience in APL is that targeting of an oncprotein through
BET INHIBITORS IN INFLAMMATION

BETi show promise as anti-inflammatory agents (57). In this study, investigators demonstrated that disruption of BRD-mediated transcription specifically interfered with the transcription of inflammation-associated genes in activated macrophages, thereby preventing the sequelae of bacterial sepsis.

several different approaches yields better responses. In APL, this has been achieved by combining retinoids with arsenic salts, which promote the degradation of the PML-RARα fusion protein (48–50). With this finding in mind, we considered other ways to target BRD-NUT. Most effective antiproliferative small-molecule inhibitors bind to and competitively inhibit highly structured, catalytic components of enzymatic oncoproteins, such as the tyrosine kinases c-Kit (51), EGFR (52), BCR-ABL (53), and B-Raf (52, 54). The bromodomains and ET domain of BRD4 are highly structured (Figure 2), which prompted Bradner and coworkers (55) to test a new class of inhibitors that bind competitively to the acetyl-histone binding pocket of BET-family protein bromodomains (i.e., BRD3, BRD4, BRDT) (Figure 10a). These BET inhibitors (BETi; see the sidebar) caused rapid dissolution of BRD4-NUT nuclear speckles, presumably by interfering with its ability to bind acetylated chromatin (14), and resulted in rapid differentiation and growth arrest of cultured NMC cells (Figure 10b). This prodifferentiative and antiproliferative activity of BETi was

Figure 10
BET inhibitor (BETi) structure and activity in NUT midline carcinoma (NMC). (a) (Left) Structure of the BETi JQ1, an active enantiomer that binds directly to the active site of BRD4, competitively with the acetylated histone tail. (Right) A space-filling representation of the high-resolution crystal structure of the first bromodomain of BRD4 bound to JQ1 (yellow). (b) Differentiation of NMC cells cultured in vitro with JQ1 over 48 h, as demonstrated by immunocytochemistry with an antikeratin (AE1/AE3) antibody. Images are shown at identical magnifications. (c) Tumor volume and survival are shown over time for cohorts of NMC tumor-bearing mice treated with JQ1 (50 mg kg$^{-1}$ daily for 18 days) or vehicle. Adapted from Reference 55.
also observed in vivo in two NMC xenograft models (Figure 10c), demonstrating the potential of BETi as a targeted NMC therapy (55). The prodifferentiative effect of BETi, as with HDACi and BRD-NUT knockdown, indicates that it is also likely to interfere with BRD-NUT function, but how is unclear. In particular, BETi are not expected to decrease the association of BRD4-NUT with p300; if this hypothesis proves true, it throws the sequestration model of BRD-NUT function into question and suggests that other modes of action will need to be considered. In this regard, it has not yet been determined whether the effect of BETi on NMC cells is purely due to interference with BRD4-NUT, or whether inhibition of normal BRD4 (expressed from an intact allele) also plays a role. If BRD4 inhibition does contribute to the response, NMC cells may be more sensitive than non-NMC cells or normal tissue because these cells are haploinsufficient for BRD4.

Another potential drawback of BETi therapy is that currently available molecules of this type bind all members of the BET family, including BRD2, BRD3, BRD4, and BRDT (55). Despite this concern about “off-BRD4-NUT” activity, however, initial studies with BETi have produced no overt toxicity in mice (55).

As in APL, in which arsenic salts and retinoids are a very potent and effective therapeutic combination, it will be of interest to determine the effects of combined HDACi and BETi therapy. Lessons from other cancers have taught us that such combination therapies will probably be essential if NMC is to be treated effectively. For example, even APL, whose fusion oncprotein, PML-RARα, is targeted with all-trans retinoic acid, cannot be cured without the addition of other agents such as arsenic (48–50, 56). In the near future, additional molecular insight into the pathogenesis of NMC will hopefully yield new clinical trials of rational targeted inhibitors.

**SUMMARY POINTS**

1. NMC is a genetically defined, highly aggressive, and incurable squamous cell carcinoma associated with chromosomal rearrangements of NUT, most commonly resulting in BRD4-NUT fusion oncogenes or, less commonly, BRD3-NUT or NUT-variant fusion oncogenes.
2. The karyotype of NMCs is uniquely simple compared with that of other squamous cell carcinomas, suggesting that it arises through a genetic or epigenetic shortcut to the phenotype of squamous cell carcinoma.
3. Once a difficult diagnosis to make, NMC is now diagnosable in most cases by immunohistochemical staining with an anti-NUT monoclonal antibody.
4. BRD-NUT chimeric oncproteins include the acetyl histone–binding bromodomains and the protein-binding ET domain of BRDs, as well as an acidic domain in NUT that binds p300.
5. BRD-NUT fusion proteins block differentiation, possibly by sequestering p300 and thereby repressing the expression of genes that are required for squamous differentiation.
6. HDACi and BETi reverse the effects of BRD-NUT fusion proteins through mechanisms that remain to be elucidated.
7. Because it is newly recognized and rare, NMC is a so-called orphan cancer for which there is now a resource for doctors, families, and patients: the International NMC Registry (http://www.NMCRegistry.org).
FUTURE ISSUES

1. The advent of targeted inhibitors of NMC may lead to more effective therapy, and clinical trials with these drugs are anticipated to occur in the near future.

2. Because the genomic localization of BRD-NUT is critical to its function, as evidenced by its inhibition by BETi, identification of the DNA with which BRD-NUT associates is imperative.

3. Cytogenetic analysis of NMC indicates that there may be relatively few collaborative background mutations, which makes NMC unique among aggressive carcinomas and, potentially, an outstanding example of a genetic shortcut to squamous cell carcinoma. Whole-genome or -exome sequencing offers a newly available means of ascertaining exactly how many, and which, genomic mutations exist in the background of NMCs.

4. Some evidence suggests that NUT-variant NMC patients may live longer than BRD-NUT NMC patients. Segregation of NUT-variant tumors into good-prognosis and poor-prognosis groups, by the identification of NUT-variant partner genes, will be key to improving our clinical and biologic understanding of these variants.

5. Because precedent predicts that NMC, like most aggressive carcinomas, cannot be cured with a single drug, clinical trials that employ a combinatorial approach with one or more targeting agents and chemotherapy may be the only way to determine its cure.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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LITERATURE CITED


30–32. Show that BRD4 binds P-TEFb to facilitate transcriptional elongation of early postmitotic genes.


43–46. Identify the mechanism of p300/CBP sequestration in disease.