Demystified molecular pathology of NUT midline carcinomas

Christopher A French

ABSTRACT

NUT midline carcinoma (NMC) is a rare, highly lethal cancer that occurs in children and adults of all ages. NMCs uniformly present in the midline, most commonly in the head, neck or mediastinum, as poorly differentiated carcinomas with variable degrees of squamous differentiation. This tumour is defined by rearrangement of the nuclear protein in testis (NUT) gene on chromosome 15q14. In most cases, NUT is involved in a balanced translocation with the BRD4 gene on chromosome 19p13.1, an event that creates a BRD4−NUT fusion gene. Variant rearrangements, some involving the BRD3 gene, occur in the remaining cases. NMC is diagnosed by detection of NUT rearrangement by fluorescence in situ hybridisation or reverse transcriptase PCR. Due its rarity and lack of characteristic histological features, most cases of NMC currently go unrecognised.

NUT MIDLINE CARCINOMA IS DEFINED MOLECULARLY

NUT midline carcinoma (NMC) is defined as any malignant epithelial tumour with rearrangement of the nuclear protein in testis (NUT) gene. In approximately two-thirds of cases, NUT (chromosome 15q14) is fused to BRD4, on chromosome 19p13.1, forming the BRD4−NUT fusion gene (figure 1A,B).1 In the remaining one-third of cases, the partner gene is BRD3 or other uncharacterised genes; we term these NUT-variant fusion genes (figure 1B).2 The NUT promoter is active only in adult testis and ciliary ganglion,1,2 and as a result only one of the two fusion genes (eg, BRD3−NUT or BRD4−NUT) is expressed. The histological features of NMC (discussed further below) are not distinctive, and diagnosis is based on detection of a NUT rearrangement by either FISH2,3 (figure 1C) or reverse transcriptase (RT)-PCR.4 NUT rearrangements also appear to be restricted to NMCs, and for this reason the diagnosis is never in question once rearrangement of NUT has been demonstrated. Presently, clinical research laboratories do not offer molecular tests for NUT rearrangement, and most testing is confined to a handful of research laboratories. Furthermore, awareness of the NMC is only now starting to spread, and there are likely to be hundreds of missed diagnoses yearly.

DEMOGRAPHICS

NMCs are rare, but their exact frequency is unknown. NMC constituted 7% of a cohort (n=98 tumours) of poorly differentiated carcinomas occurring in people under the age of 40 years.5 In a more recent study that was not restricted to young adults and children, NMC made up 18% of poorly differentiated carcinomas of the upper aerodigestive tract, particularly the sinonasal region.6 Importantly, the average age of patients with NMC in that study was 47 years, overturning the perception that NMCs are largely confined to children and young adults.6 As indicated in table 1 (a summary of all 22 cases reported to date), the age at presentation ranges from 5 to 78 years, and there is a suggestion of a female predominance (M:F 0.69). The young age at presentation (mean age 25 years) may reflect selection bias generated by clinical patterns of cytogenetic analysis, as karyotypes are obtained frequently on solid tumours in children and rarely on adult carcinomas.

CLINICOPATHOLOGICAL FEATURES OF NMCS

The clinicopathological features of NMCs are summarised in table 1. Most NMCs present in the midline of the upper aerodigestive tract or the mediastinum. The lung is sometimes involved when tumours are large in size, possibly due to secondary extension; there are no proven examples of primary tumours arising in the lung. A few unusual cases have presented below the diaphragm, including tumours that presented in the posterior bladder and the bones of the pelvis, respectively. No cases outside of the midline axis have been reported.

With the exception of one case,6 all patients with NMCs have died (table 1). The mean survival is less than 1 year (9.5 months) despite aggressive chemotherapy and radiation treatment. Most deaths have been caused by local effects of tumour, or complications of treatment. Early data3 suggested that patients with NUT-variant NMCs survived longer than those with BRD4−NUT rearrangements (cases 9−11, table 1), but subsequent cases (case 17, table 1, and author’s unpublished findings) have suggested that there is no difference in survival between these two molecular classes of NMC. The lethality of NMC and the existence of a highly characteristic molecular lesion provide a compelling rationale for attempting to develop therapies that target NUT fusion proteins.

The one patient with a BRD4−NUT NMC who has survived6 is atypical in three respects. First, and most notably, this patient is the sole apparent cure reported among patients with this tumour. Second, the tumour arose within the bones of the pelvis (case 3, table 1); and third, it lacked epithelial differentiation, as judged by electron microscopy and immunohistochemistry (IHC). Based on the absence of epithelial features, the original diagnosis was Ewing sarcoma without EWS rearrangement, and the patient was treated with the Scandinavian

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Sarcoma Group (SSG) IX protocol for inoperable Ewing sarcoma. Based on this anecdotal evidence and the failure of NMCs to respond to other therapeutic regimens, several patients with NMC have been treated with Ewing sarcoma protocols subsequently (author’s unpublished observations), but all have succumbed to their disease. It thus seems likely that the fortunate outcome in the one survivor of NMC was due to the unusual characteristics of the tumour, and not due to fortuitous treatment with a regimen that is generally effective in NMC.

**CELL OF ORIGIN**

NMCs were initially thought to be derived exclusively from thymus, but Table 1 describes the clinical characteristics of reported NUT midline carcinomas.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Location</th>
<th>Diagnosis</th>
<th>Survival (months)</th>
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<th>NUT-variant</th>
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<td>French et al</td>
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<tr>
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<td>Undifferentiated carcinoma</td>
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</table>

AV/0D, alive without disease; NK, not known; PD carcinoma, poorly differentiated carcinoma; PDSQC, poorly differentiated squamous cell carcinoma; PNET, primitive neuroectodermal tumour; SNUC, sinonasal undifferentiated carcinoma; SQC, squamous cell carcinoma.

**Figure 1** Translocations of the nuclear protein in testis (NUT) gene are pathognomonic of NUT midline carcinomas (NMCs). (A) Karyotype of a patient with BRD4—NUT NMC is remarkably simple, and reveals a reciprocal translocation between NUT, on 15q14, and BRD4, on 19p13.1 (t(15;19)(q14;p13.1)). Red and green dots illustrate where split-apart fluorescence in situ hybridisation (FISH) probes flanking NUT (green, telomeric to NUT; red, centromeric to NUT) can be used for diagnostic FISH (as seen in (C)). (B) Schematic of the fusion oncogenes formed when NUT is translocated to BRD3 and BRD4 loci (adapted from French et al) reveals that the predicted oncoproteins contain both bromodomains and the extra terminal domain of BRD3/BRD4, and nearly the entirety of NUT. (C) Diagnosis of a NMC can be made using a dual-colour FISH split-apart assay of differentially labelled probes flanking the NUT gene (see (A) above) on formalin-fixed paraffin sections of tumour (adapted from French et al).
numerous non-thymic tumours, including tumours arising below the diaphragm, have been reported subsequently. Because of the frequent involvement of midline structures in the head, neck, mediastinal and other midline structures, we speculate that NMCs arise from primitive neural crest-derived cells. This is in line with the absence of any in situ component when tumours involve epithelium-lined organs (eg, trachea or epiglottis), and it is also consistent with expression profiling results, which show the highest level of expression in the adult ciliary ganglion (BioGPS, http://biogps.gnf.org/), a neural-crest-derived structure.

PATHOGENESIS
As is true of most other translocations associated with specific human cancers, the basis for the genesis of NUT rearrangements is unknown. There are no known associations with exposures to environmental toxins or infectious agents, smoking, or oncogenic viruses such as human papillomavirus or Epstein–Barr virus® (Stelow et al® and author’s unpublished observations). Transforming activity by NUT-fusion proteins has not been demonstrated directly, but all karyotypes thus far obtained have been remarkably simple (such as acute leukaemias), this association suggests that NUT rearrangements are a very early, possibly tumour-initiating, event.

The normal function of BRD4 is beginning to be elucidated. BRD4 was originally named mitotic-chromosome-associated protein for its unique property of remaining bound to chromatin, via its two bromodomains, during mitosis.® By means of its mitotic-chromosome binding, BRD4 is hypothesised to mark actively transcribed genes before mitosis, thus providing a kind of cellular memory that ensures re-initiation of transcription from these sites after mitosis is completed. Two recent studies have provided evidence that this may indeed be the case. By recruiting cdk9 (a component of the P-TEFb transcription elongation complex) and RNA polymerase II to chromatin during late mitosis, BRD4 initiates the transcription of G1 genes.®

The function of BRD3 is less characterised but, like all proteins in the BRD family, it also contains two acetyl-histone-binding bromodomains and the obscure extra terminal domain.® BRD3 is highly homologous to BRD4 (figure 1A), and so its involvement in NMCs is no surprise.

In contrast to BRD proteins, NUT is likely to be unstructured and lacks known functional domains. Further, unlike the BRD proteins, NUT is poorly conserved and apparently restricted to mammals, as no homologue has been identified in lower vertebrates. For these reasons, little is currently known about NUT. Unlike BRD4 or BRD3, NUT normally shuttles between the nucleus and cytoplasm, but it remains bound to chromatin when fused to BRD4 or BRD3.® This has led to the hypothesis that the BRD protein moiety in the fusion proteins serves to tether NUT to chromatin, thus modifying the function of either or both proteins in a way that affects transcription. One important consequence of BRD—NUT expression has been discovered by using siRNA to knock down the expression of BRD3–NUT or BRD4–NUT in NMC cell lines. Withdrawal of the NUT fusion proteins resulted in a dramatic and irreversible squamous

Table 2  Morphological mimics of NUT midline carcinoma

<table>
<thead>
<tr>
<th>Tumour</th>
<th>Monomorphic</th>
<th>Distinguishing marker*</th>
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<tr>
<td>Basaloid squamous cell carcinoma</td>
<td>—</td>
<td>Human papillomavirus®–22</td>
</tr>
<tr>
<td>Ewings/primitive neuroectodermal tumour</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Extragonadal germ cell tumour</td>
<td>+</td>
<td>PLAP, Oct 3/4, β-HCG</td>
</tr>
<tr>
<td>Lymphoma/leukaemia</td>
<td>+</td>
<td>Leucocyte common antigen</td>
</tr>
<tr>
<td>Nasopharyngeal carcinoma</td>
<td>+</td>
<td>Epstein–Barr virus</td>
</tr>
<tr>
<td>Non-NMC PDSQC</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Olfactory neuroblastoma</td>
<td>+</td>
<td>Synaptophysin</td>
</tr>
<tr>
<td>Poorly differentiated carcinoma</td>
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<td>—</td>
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<tr>
<td>Small cell carcinoma</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>SNUC</td>
<td>+</td>
<td>—</td>
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</tbody>
</table>

*All the markers are negative in NMCs (French CA et al® and author’s unpublished observations).

β-HCG, β human chorionic gonadotrophin; NMC, NUT midline carcinoma; PDSQC, poorly differentiated squamous cell carcinoma; PLAP, placental-like alkaline phosphatase; SNUC, sinonasal undifferentiated carcinoma.

Figure 2  BRD4—NUT blocks differentiation. (A) 72 h after negative control siRNA transfection of the BRD4—NUT-expressing cell line TC-797, the cells remain undifferentiated and unchanged (bar 50 μm) (adapted from French et al®). (B) After siRNA-induced BRD4—NUT knockdown, the TC-797 cells display dramatic squamous differentiation, with stratification and enlargement of cells (adapted from French et al®). (C) 3 weeks after siRNA knockdown, TC-797 cells resemble benign, parakeratotic squamous epithelium, with flattening of cells, and condensation of their nuclei.
differentiation and arrested growth (figure 2). These findings, which are reminiscent of the effects of retinoic acid on the differentiation of acute promyelocytic leukaemia cells expressing PML–RARα fusion proteins, demonstrate that BRD–NUT proteins block differentiation. The dramatic phenotype produced by BRD–NUT knockdown offers an excellent readout for high-throughput screens of drugs that interfere with BRD–NUT fusion protein functions.

**DIAGNOSIS**

The diagnosis of NMC is made definitively by demonstration of NUT rearrangement by fluorescence in situ hybridisation (FISH) (figure 1C) or by demonstration of a BRD4–NUT fusion transcript by RT-PCR. FISH is preferred because it will detect all NMCs, including all NUT variants, whereas RT-PCR can currently only detect BRD3–NUT or BRD4–NUT tumours. The challenge is not the diagnosis of NMC, but to determine when to perform the test, which is not yet available in clinical laboratories.

If NMC is not to be missed, any poorly differentiated, monomorphic, midline neoplasm that does not stain for lineage-specific markers should be considered for NUT-rearrangement testing (table 2, figure 3A–G). This group includes sinonasal undifferentiated carcinomas and any poorly differentiated carcinomas.

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**Figure 3** NUT midline carcinomas (NMCs) are monomorphic, poorly differentiated carcinomas with varying degrees of squamous differentiation. (A, B) NMCs can mimic other undifferentiated neoplasms such as germ cell tumours, Ewing sarcoma or lymphoma. (C, D) Abrupt squamous differentiation is characteristic of some NMCs. (E, F) NUT-variant NMCs look similar to BRD4–NUT tumours. (G) Rarely, there can be extensive squamous differentiation. (H, I) Poorly differentiated, non-NMCs often display pleomorphism and atypia that is uncharacteristic of NMCs.

**Figure 4** NUT polyclonal antibody stains testis and NUT midline carcinoma (NMC). (A) The spermatogonia and maturing spermatocytes, but not sperm, of testis produce NUT. Diffuse nuclear reactivity with NUT antibody is seen in (B), a BRD4–NUT tumour, (C, D) two BRD3–NUT tumours, and (E–G) three uncharacterised NUT-variant tumours, (H) but not in this squamous cell carcinoma without NUT rearrangement. (D, inset) A speckled nuclear pattern of NUT antibody staining is characteristic of NMCs. (B–H) Adapted from French et al. 2
NMC is an aggressive carcinoma genetically defined by rearrangement of NUT.
- NMC is likely vastly under-recognized and under-diagnosed.
- Most NMCs are squamous cell carcinomas and can only be identified by molecular or immunohistochemical testing.
- The diagnosis of NMC should be considered in any non-smoking patient with poorly differentiated squamous cell carcinoma.

Demystified

Take-home messages

- Focal squamous differentiation is common in NMCs (figure 3C, D, F), and may occasionally be extensive (figure 3G).
- A curious, characteristic finding is focal abrupt squamous differentiation (figure 3C, D), where stratification and gradual differentiation are absent. To our knowledge, abrupt squamous differentiation, while common in NMCs, is rarely seen in other carcinomas.
- As is the case for certain other oncoproteins, such as cyclin D1 and anaplastic lymphoma kinase, that are mis-expressed due to chromosomal translocations, it is possible that IHC tests for NUT will prove to be valuable aids in the diagnosis of NMC.
- Normal NUT expression is restricted almost exclusively to the testis (figure 4A). Thus, positive nuclear IHC staining for NUT in tissue outside the testis is indicative of aberrant expression, such as in NMCs, where both BRD4–NUT and NUT-variants localise to the nucleus (figure 4B–G). We have tested our own non-commercial rabbit polyclonal NUT antibodies for diagnostic utility using a panel of five NMCs and 23 NUT-unrelated poorly differentiation carcinomas of the upper aerodigestive tract. We found that our NUT antibody has a specificity of 95% and a sensitivity of 60%, indicating that it falls short of the sensitivity one would like in a diagnostic test that is envisioned as a screen for the selection of tumours for confirmatory FISH testing. It is hoped that NUT monoclonal antibodies, which are currently being evaluated, will permit the development of a more sensitive IHC-based diagnostic screening test.

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