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NUT Midline Carcinoma

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Abstract

NUT midline carcinoma (NMC) is a rare, genetically defined, aggressive human cancer defined by rearrangements of the gene NUT. In the majority (approximately 75%) of NMCs most of the coding sequence of NUT on chromosome 15q14 is fused BRD4 or BRD3, creating chimeric genes that encode BRD-NUT fusion proteins. In the remaining cases, NUT is fused to an unknown partner gene; these tumors are termed NUT-variant. Diagnosis of NMC is made by demonstration of expression of the NUT-fusion protein using a monoclonal antibody to NUT for immunohistochemistry, and confirmation of the fusion (BRD-NUT or NUT-variant) by fluorescent in situ hybridization or RT-PCR. Because the reagents and expertise required to diagnose NMC are not available in most laboratories, and the incomplete awareness of this disease, NMC is frequently undiagnosed or misdiagnosed, and its actual prevalence is unknown. NMC does not arise from any specific tissue type or organ. It presents as a poorly differentiated carcinoma originating from midline locations such as the head, neck or mediastinum. Although rare, NMCs occur throughout life and advanced local disease is frequently accompanied by distant hematogenous metastases. There remains no effective treatment for NMC, there are no guidelines, and current approaches to treatment are based on discussions amongst a few oncologists who each have had a single experience treating this disease. BRD-NUT functions to block cellular differentiation and promote uncontrolled growth of carcinoma cells.

Keywords

BRD4; NUT; BRD4-NUT; t(15; 19); carcinoma

History

In the summer of 1999, a 12 year old girl, who was to become the first American patient with NUT midline carcinoma, developed a sore throat and difficulty swallowing, and eventually developed a muffled voice. There was no response to antibiotics, and a month later was seen by an otolaryngologist. Upon examination, an ulcerating mass was found to have replaced her epiglottis. The biopsy pathology resembled that of a nasopharyngeal carcinoma. She was treated accordingly, at Children's Hospital, Boston, and enjoyed an initial shrinkage of her tumor, but the tumor came back and she died a horrible death, strangled by the tumor which closed off her airway. The clinicians would not forget this tumor, but the abrupt end to her life marked the discovery of a new type of cancer.

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It was fortunate that the pathologists at Children's Hospital had sent some of the patient's initial biopsy to Dr. Paola Dal Cin of Brigham and Women's Hospital for cytogenetic analysis. She found that this was no ordinary carcinoma, a class of solid tumors characterized by notoriously complex karyotypes. Apart from a trisomy 8, the patient's tumor's 47 xx karyotype harbored one distinct abnormality, a t(15;19)(q13;p13.1). The news of this karyotype was noticed by Dr. Jonathan Fletcher, who pays attention to rare, translocation-associated solid tumors. His review of the literature revealed the existence of three isolated case reports of pediatric thymic carcinomas with associated t(15;19)s, all aggressive and rapidly lethal, a clinical course which resembled that of the index patient [1]. From one of these cases, which originated in Japan, Dr. Kubonishi had created a cell line [2], Ty-82. With the purpose of cloning the presumed fusion oncogene resulting from the t(15;19) that was hypothetically responsible for creating this unusual and aggressive carcinoma, Dr. Fletcher acquired the cell line and handed over the gene mapping project to a very green yet eager post-doctoral fellow, this author.

Breakpoint mapping back then, 2000, had been made much easier with the newly available YAC and cosmid libraries that could be used to FISH-map rapidly. This was done for both the 15q13 and 19p13.1 loci in less than a year, and the loci were finely mapped by southern analysis. The chromosome 19 locus was located in the middle of a newly described gene, BRD4 [3], and the chromosome 15 locus appeared to be very close to a small gene, Nop10p. Attempts to demonstrate that the Nop10p gene was present in a fusion transcript with BRD4 failed, however, and around that time a few new ESTs appeared within the area on the UCSC genome browser. Having failed to RACE out a product, we attempted RT-PCR using primers to these novel ESTs, and quickly succeeded at amplifying an in-frame fusion transcript between BRD4 and these ESTs. The ESTs collectively formed a putative 3.5kb gene that we initially called "PRUC", for proline-rich in undifferentiated carcinoma. Unimpressed with this term, and having observed that it appeared to be expressed only in testis, as demonstrated in multiple tissue northern blotting [4], we chose a more appropriate acronym, NUT, for Nuclear protein in testis. That name has since been changed to "chr15orf55" by the consensus of nomenclature rules, but we still use the name NUT. Hence, the name *BRD4-NUT* for this fusion oncogene remains unchanged.

In the course of FISH-mapping the chromosome 19 and 15 breakpoints, we had developed some robust probes that could be used to screen new and archival tumors in search of more cases of what we termed, "t(15;19) carcinomas". Our criteria were broad. We looked at all poorly differentiated carcinomas in patients younger than forty years of age, ignoring, for the time, adult carcinomas which were predicted to harbor 100s of mutations and complex karyotypes, a pathogenetic mechanism unlikely to overlap that of the t(15;19) carcinoma. We found seven tumors with *NUT* rearrangements within 98 tumors screened [5]. What was interesting was that while four of these were *BRD4-NUT* tumors, three had rearrangement of *NUT*, but not *BRD4*, termed *NUT-variants*. This has led to the hypothesis that the common denominator was *NUT*, and not *BRD4*. Hence, after several iterations, the current name of this cancer is NUT midline carcinoma, or NMC, to reflect that it is defined by chromosomal rearrangement of *NUT*.

Clinico-pathologic features

To date, we have collected enough cases of NMC, screened mostly by FISH, to be able to roughly describe the disease as follows. While likely rare, the actual frequency of NMC has not been determined. As above, 7% of 98 mostly carcinomas in patients less than 40 were NMCs [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

this study was 47, overturning the perception that NMCs are largely confined to children and young adults [5]. Parenthetically, many recent patients in this author's experience have been adults over the age of 30.

The clinico-pathologic features of NMCs are summarized in table 1 [7]. The age at presentation ranges from 3 to 78 years. The young age (avg. age 25) is likely distorted by selection bias in the original study as described above [5]. NMC is a genetically defined disease and does not arise from a specific organ. Most cases occur in the mediastinum and upper aerodigestive tract, but exceptional tumors have arisen in bone [16], the bladder [5], the abdominal retroperitoneum, the pancreas, and the salivary glands [17,18].

With the exception of one case [16], all patients with NMCs have died (table 1). The average survival is less than one year (9.5 mo.) despite aggressive chemotherapy and radiation treatment. Some data [5] and unpublished observations suggests that patients with *NUT*-variant NMCs may live longer than those with *BRD4-NUT* rearrangements (cases 9–11, table 1); this has yet to be formally demonstrated in a larger series.

Molecular Cytogenetics

Perhaps the most unique feature of NMCs are their simple karyotypes. The tumors often harbor only a single abnormality, the t(15;19)(q14;p13.1), and in this way more resemble leukemia than carcinoma, again pointing to the likely critical biologic importance of the fusion oncogene. Variants which create the *BRD4-NUT* fusion have included more complex three-way translocations [12] and unpublished observations, and some tumors have had cryptic *NUT* breakpoints undetectable by conventional FISH [19].

In approximately 2/3rds of cases, *NUT* (chromosome 15q14) is fused to *BRD4*, on chromosome 19p13.1, forming the *BRD4-NUT* fusion gene (fig 1) [4]. The remaining 1/3rd of cases are *NUT-vairants* where the partner gene is *BRD3* or other uncharacterized genes [20]. The *NUT* promoter is active only in adult testis and ciliary ganglion [4,20], and as a result, only one of the two fusion genes (e.g., *BRD-NUT*, but not *NUT-BRD*) are expressed. The breakpoints themselves characteristically occur within intron 10 of *BRD4*, or intron 9 of *BRD3*, and intron 2 of *NUT*, fusing the business end of *BRD4*, encoding both acetyl-histone binding bromodomains and extraterminal domain, with virtually the entire *NUT* gene.

Diagnosis

The histologic features of NMC are, unfortunately, not diagnostic. The morphology is that of a poorly differentiated carcinoma, with or without squamous differentiation. It does have a distinctly monomorphic, clonal appearance, as contrasted with the garden variety poorly differentiated carcinoma, which tends to be pleomorphic. NMC is a new disease, and is not broadly known to most pathologists nationally and internationally. It is therefore commonly undiagnosed, or misdiagnosed, most often as squamous cell carcinoma, but occasionally as other specific pathogenetic entities, such as Ewing sarcoma or Sinonasal undifferentiated carcinoma (SNUC). The result is that the actual frequency of NMC is unknown, and likely much more common than currently thought to be.

The diagnosis of NMC has historically been made by demonstration of *NUT* rearrangement by dual color, split-apart FISH using probes flanking *NUT*, or by demonstration of a *BRD4-NUT* fusion transcript by RT-PCR [15]. FISH is preferred because it will detect all NMCs, including all *NUT*-variants, whereas RT-PCR can currently only detect *BRD3-* or *BRD4-NUT* tumors. Because these assays are generally not available in most pathology or molecular diagnostic laboratories, we sought to develop a diagnostic monoclonal antibody to *NUT*, taking advantage of the fact that the native protein is not expressed outside of the

testis. Staining a large panel of common carcinomas (n~1000), and including 30 FISH-positive NMCs, with the NUT antibody by immunohistochemistry, we found that it had a sensitivity of 87%, and a specificity of 100% [19]. We now use this antibody (Cell Signaling Technologies, Inc., Danvers, MA) routinely for diagnosis. The advent of this new immunohistochemical test for NMC makes diagnosis easy in any pathology laboratory, and due to the new user-friendliness of the diagnosis, it is expected that NMC will be added to the differential diagnosis of all non-smoking-related poorly differentiated carcinomas. Consequently, a much more accurate measure of the frequency of this disease is predicted to emerge in the near future.

Now, the challenge is not the diagnosis of NMC, but when to perform the immunohistochemical test for NUT expression. A useful guideline is to consider NMC in the differential diagnosis of any midline poorly differentiated carcinoma in a never-smoking patient, and in the absence of EBV or HPV within the tumor.

BRD4, NUT, and BRD4-NUT Function

BRD4 is known to bind acetylated histones, which are associated with actively transcribed DNA, including those of mitotic chromosomes [21,22]. Through its constitutive association with active chromatin, as well as with the transcriptional elongation complex, pTEFb, BRD4 is thought to preserve cellular memory by marking regions for re-initiation of transcription following mitosis [23–26]. *NUT* encodes an unstructured polypeptide expressed only in post-meiotic spermatids, but whose function is otherwise unknown.

Involvement of BRD4 and possibly NUT in chromatin regulation suggested that BRD4-NUT might somehow modify chromatin so as to prevent the expression of genes needed for epithelial differentiation. Indeed, as with PML-RAR α in acute promyelocytic leukemia, abrogation of BRD4-NUT or BRD3-NUT activity in NMC cell lines through siRNA-mediated knockdown results in induction of squamous differentiation followed by growth arrest (Fig. 2, [20]). Long term studies (3 weeks) have indeed found that the differentiation is terminal and irreversible. The findings suggest that BRD4-NUT is responsible for blocking differentiation and maintaining the cells in a perpetual state of proliferation, and that BRD4-NUT is potentially a powerful therapeutic target.

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FIG 1.
 Cartoon of BRD4, BRD3, NUT, and BRD-NUT fusion proteins. ET, extraterminal domain.
 NLS, nuclear localization signal. NES, nuclear export signal.

FIG 2.

Knockdown of BRD4-NUT results in squamous differentiation and growth arrest (from [20]). (A-C) Control siRNA (ct) has no effect on the BRD4-NUT-expressing cell lines TC797 and PER-403 [10], or the BRD3-NUT-expressing cell line 10326, resp. (hematoxylin and eosin stain, 400x, 96h post-transfection). Morphologic changes induced by NUT siRNA-induced knockdown (kd) in TC797, PER-403, and 10326 cells (hematoxylin and eosin stain, 400x, 96h post-transfection). (A-C, insets) BRD-NUT knockdown induces keratin expression, a biomarker of epithelial differentiation, as evidenced by increased staining for pan-keratin monoclonal antibody (clone MNFI16, DAKO). (D) Knockdown of BRD-NUT decreases proliferation. Staining for Ki-67+ (a marker of cell cycle progression) was performed on sections of cell blocks prepared 96h post-siRNA transfection. Bars = 50 μ m, A-C. (E) Effects of a *NUT*-specific siRNA on BRD-NUT. Protein extracts obtained 24h after siRNA transfection were analyzed on a Western blot stained with a polyclonal NUT antibody.

Table 1

Clinical characteristics of reported NUT midline carcinomas (from [7]).

Case no.	Age (yr.)	sex	Location	Diagnosis	Survival (mo.)	Brd4-NUT	NUT-variant	Ref.
1	3	M	Bladder	PD sq cell ca	8.5	✓		[5]
2	5	M	Mediastinum	Mucoepidermoid ca	3.5	✓		[8]
3	10	M	Iliac bone	Ewing's sarcoma/PNET	AW/OD 180*	✓		[9]
4	11	F	Thorax	Undiff. ca	4.5	✓		[10]
5	12	F	Nasopharynx	PD sq cell ca	3.25	✓		[1,11]
6	13	F	Epiglottis	Undiff. ca	9	✓		[1]
7	15	M	Mediastinum	Thymic PD sq cell ca	6	✓		[12]
8	15	F	Orbit	PD sq cell ca	7	✓		[5]
9	16	M	Lung	Sq cell ca	37		✓	[5,13]
10	16	F	Trachea	PD ca	31.5		✓	[5]
11	21	F	Nasopharynx	Nasopharyngeal ca	10.25		✓	[5]
12	22	F	Thymus	PD ca	3.5	✓		[14]
13	26	M	Sinonasal	Undiff ca	16.75	✓		[5]
14	30	F	Mediastinum	PD ca	3.25	✓		[15]
15	31	M	Nasal Cavity	SNUC		✓		
16	34	F	Thorax	NK	NK	✓		[9]
17	34	M	Mediastinum	PD ca	2		✓	[13]
18	35	F	Mediastinum	PD ca	2	✓		[5]
19	39	F	Nasal cavity and sinus	PD sq cell ca		✓		
20	40	F	Nasal cavity and sinus	PD sq cell ca		✓		
21	47	M	Nasal cavity and sinus	SNUC		✓		
22	78	F	Supraglottic larynx	Undiff ca		✓		
Avg.	25.14				9.53			