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Diagnosis of NUT Midline Carcinoma Using a NUT-Specific Monoclonal Antibody

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INTRODUCTION

NUT midline carcinoma (NMC) is a recently recognized cancer uniquely defined by the presence of chromosomal rearrangements involving the *NUT* (Nuclear protein in testis) gene on chromosome 15q14 (5). In approximately 80% of cases the chromosomal translocation occurs between *NUT* and *BRD4* on chromosome 19, resulting in the formation of a *BRD4-NUT* fusion gene. In the remaining tumors, variant NUT rearrangements are present involving *BRD3*(5), which is highly homologous to *BRD4-NUT*, or other unknown partners. *BRD4-NUT* and *BRD3-NUT* encode fusion proteins that appear to contribute to carcinogenesis by blocking epithelial cell differentiation (5).

NMCs are aggressive and highly lethal, with an average survival of less than one year(1). Although NMC is likely to be a rare cancer, it is a newly recognized entity that is morphologically indistinguishable from other poorly differentiated carcinomas, and thus its true incidence is unknown. One recent report found that amongst poorly differentiated carcinomas in non-smokers, predominantly of the upper aerodigestive tract, its prevalence ranges from 7 to 20%(1,8). Initially thought to be a childhood cancer, it has recently been shown that NMC affects people of all ages(8); there is no predilection for either sex.

Based on the poor response of NMC to chemotherapy regimens designed to treat carcinomas and the cure of one patient with NMC using a chemotherapeutic regimen designed for Ewing sarcoma(6), there has been a move towards treatment of NMC with variations of the Euro Ewing 99 sarcoma protocol (unpublished observations). This has led to an increased interest in the accurate and timely diagnosis of NMC. Currently, NMC is usually diagnosed by FISH

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using *NUT* split-apart probes(2), but this test is not widely available and has not been commercialized. Thus, there is a need for a simple, reliable diagnostic test for NMC.

NUT expression is normally confined to the germ cells of the testis(4) and ovary (reported here) and has not been detected in human tumors other than NMC. This suggested that it should be possible to develop a diagnostic IHC test for NMC with a NUT-specific antibody. A polyclonal rabbit antiserum raised against NUT gave promising results, but was not sensitive or specific enough to be an ideal diagnostic reagent, in part due to cross-reactivity with other antigens(8). Therefore, we sought to raise monoclonal antibodies to NUT for purposes of diagnostic test development.

MATERIALS AND METHODS

NUT Monoclonal Antibody Production

A GST fusion protein containing amino acids 450–700 of human NUT was used to immunize New Zealand rabbits (Cell Signaling Technology, Inc. (CST), Danvers, MA). Positive immuno-reactive rabbits were identified by Western blotting and IHC and chosen for rabbit monoclonal development. Three lead monoclonal antibodies were chosen for further clinical validation. The NUT antibody is being prepared for commercial release and will be available from CST.

Cell lines

The BRD4-NUT-expressing cell line, TC797, has been described previously(9). All other lines were obtained from the American Type Culture Collection (Manassas, Va.). TC797 and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA.) supplemented with a solution containing 10% bovine growth serum (Hyclone, Logan, Utah), 2 mM L-glutamine (Gibco), 100 U of penicillin G/ml, and 100 mg of streptomycin/ml. The A549, A673 and MCF7 cell lines were acquired through ATCC and grown as recommended by the supplier.

Expression plasmids, siRNA, and transient transfection

A cDNA encoding FLAG-BRD4-NUT was assembled in the plasmid pcDNA3 (Invitrogen, Carlsbad, CA) as described(5). A small interfering RNA (siRNA) duplex designed against human *NUT*, (5'- AAACTCAGAACTTTATCCTTACCTGTCTC –3'), and scrambled siRNA (*Silencer*® Negative Control #1) were purchased from Applied Biosystems/Ambion (Austin, Tx). Transfection of pcDNA3-FLAG-BRD4-NUT into 293T cells was performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Transfection of siRNA into TC797 cells was performed by electroporation as described(5). Briefly, siRNA (680nM in 100ul solution R) was transfected into 3×10^6 cells using the Nucleofector II instrument and program T-27 (Amaxa Inc., Gaithersburg, MD).

Fluorescence *in situ* Hybridization

Dual-color FISH assays were performed on formalin-fixed paraffin-embedded 4μm tissue sections as described(3). Probes used for the 15q14 *NUT* breakpoint, flanking a 181kb region containing *NUT*, included the 3' telomeric BAC clones 1H8 and 64o3, and the 5' centromeric clones 412e10 and 3d4. Probes used for the 19p13.1 *BRD4* breakpoint were the 5' centromeric BAC clone 187l3 and the 3' telomeric BAC clone 87m17. The probe spanning NUT, BAC clone 122p18, was used to detect the cryptic NUT breakpoint in a bring-together assay with 5' centromeric BAC clone 187l3. Sections in which >80% of cells contained hybridization signals in four areas (200 cells/area) were considered adequate for interpretation.

FISH for *NUT* rearrangement was evaluable in 481 cases. This included one author's (CAF) collection of cases (N = 141, Group 1, below), a head and neck tumor microarray (N = 327, from Group 2 below), and selected testicular and ovarian germ cell tumors (N = 13, from Group 2 and 3 below).

Immunohistochemistry

IHC was performed on 5 μm sections prepared from formalin-fixed, paraffin-embedded primary tumors. To stain for NUT, following deparaffinization and rehydration, sections were subjected to antigen retrieval in Dako pH 9.0 solution (Dako USA, Capinteria, CA) in a steam pressure cooker (BioCare Medical, Walnut Creek, CA). Other antigen retrieval buffers that were tested and determined to be less effective in producing optimal signal/noise on control tissues included citrate buffer, pH6, and EDTA buffer, pH8 (both from Zymed-Invitrogen). After washing in distilled water and treatment with Peroxidase Block (Dako) for 5 min to quench endogenous peroxidase activity, sections were incubated with primary rabbit monoclonal anti-NUT (9.2ug/ml) in antibody diluent (Dako) for 1 hr, washed in 50 mM Tris-HCl (pH 7.4), and incubated with horseradish peroxidase–conjugated secondary antibodies (Envision detection kit, DAKO USA). Staining was developed through incubation with diaminobenzidine (DAB), and sections were counterstained with hematoxylin.

The results of IHC staining were interpreted independently by two pathologists (CAF and JCA), who were both blind to the FISH results. Cases were scored based on the extent of nuclear immunoreactivity in the tumor cells. Cases with unequivocal nuclear staining in the majority of the tumor cells were considered positive. Consensus was reached in all discrepant cases through dual review and discussion.

Reverse Transcriptase Polymerase Chain Reaction

RNA was extracted from fresh human peripheral lymphocytes, TC797 cells, frozen human testis, and dysgerminoma (Fig.3a) using Trizol according to the manufacturer's instructions (Invitrogen). cDNA was synthesized using ArrayScript reverse trancriptase and random decamers (Ambion/ Applied Biosystems, Inc.) according to the manufacturer's instructions. PCR was performed using primer sets A (NUT 750fwd - 5'-GCTGAAGCCCACTATGACCCTGGAG-3', NUT 994rev - 5'-TGGAGGCTGCCTTCTTCGGAATGTA-3') and B (NUT 750fwd, NUT 1289rev - 5'-TCTGCCAGAAATTGAGGGTGAATGA-3'), which cross intron 3–4 and 3–5 of *NUT*, respectively.

Samples

To make cell-blocks, cultured 797 cells transfected with either siRNA specific for NUT or control siRNA were collected by trypsinization (Invitrogen), washed with PBS, and fixed for 20 min in 10% buffered formalin. After repeated washing with PBS, fixed cell pellets were suspended in warmed Histogel according to the manufacturer's instructions (Invitrogen). After gelation was complete, the pellets were paraffin-embedded and used as controls during IHC test development.

Archival tumor sections (4μm thick, formalin-fixed, paraffin-embedded) were collected from four different sources. Group 1 (N = 141) consisted of one author's (CAF) collection of cases, all of which have been evaluated for NUT rearrangement by FISH, and includes 28 known NMCs. Group 2 consisted of several non-commercial tumor microarrays (TMA, N = 674 cases): a head and neck squamous cell carcinoma TMA (provided by EBS, N = 442 cases, 2 cores/ neoplasm), that included 327 predominantly smoking-related carcinomas; a miscellaneous carcinoma TMA (provided by EBS, N = 165 cases, 4 cores/ neoplasm); and two male germ cell tumor TMAs (provided by MAR, N = 67 cases, 2 cores/ neoplasm). Group 3

was a collection of ovarian germ cell tumors (provided by MCC, N = 34). Group 4 was a commercial TMA of common carcinomas (US Biomax MC2081 TMA, N = 208 tissues, 1 core/ neoplasm (photos of cores available at http://www.biomax.us/tmaimage.php?catalognum=MC2081); US Biomax, Rockville, MD). Studies were performed in accordance with IRB protocol 2000-P-001990/6; BWH.

RESULTS

Antibody Validation

Three lead rabbit monoclonal antibodies were initially screened by Western blotting and IHC staining of sections of cell blocks prepared from the NMC cell line 797, which bears a *BRD4-NUT4* fusion gene. One antibody, C52, specifically stained proteins of the expected size of NUT and BRD4-NUT on Western blots, and showed nuclear immunoreactivity in normal human testis and 797 cells (Fig. 1). Particularly in 797 cells, nuclear reactivity appeared in a speckled pattern similar to that previously noted with epitope-tagged BRD4-NUT(5). Immunoreactivity in 797 cells was greatly reduced by siRNA knockdown of BRD4-NUT (Fig. 1D), which produced the characteristic changes in cell size and morphology that accompany differentiation of this cell line following BRD4-NUT knockdown (5). Based on these validation studies, we proceeded to evaluate C52 staining of archival tissue collections.

Case Characteristics

The case characteristics are summarized in Table 1, Table 2, Table 3, Table 4. The total number of tissues stained with the C52 antibody, both malignant (N = 1030) and normal (N = 38), was 1068. The tumors stained were mostly carcinomas of the larynx, oral cavity and lung (Table 1). Also included were many common carcinomas, including those of the breast, prostate, ovary, colon, uterus, kidney, pancreas, and bladder. The types of tumors that were evaluated are summarized in Table 2.

Within this mix of cases, we included 28 FISH-proven NMCs as positive controls. NMCs most commonly involved the mediastinum, sinonasal region, or the lung (Table 3), and histologically most often resembled squamous cell or poorly differentiated carcinoma (Table 4).

Immunohistochemistry with NUT Antibody

The interpretation of C52 staining was straightforward in the vast majority of cases. Tumors that were positive typically revealed diffuse (>90%) strong nuclear reactivity in a speckled pattern, whereas negative cases lacked any nuclear reactivity (Fig. 2). Weak cytoplasmic staining in benign and malignant epithelial cells was not uncommon, but did not lead to any difficulties with interpretation. The cytoplasmic staining could be due to expression of endogenous NUT (5), but because prior studies have failed to detect NUT mRNA expression except in testis (4), it seems more likely to represent non-specific background staining. Among the 1068 tissues stained, there were two discrepant interpretations between the two pathologists. Both of these discrepancies occurred in FISH-positive tumors. In both instances, the tumor cells exhibited weak nuclear staining interpreted by one pathologist as positive and the second as negative. To produce a conservative estimate of the overall performance of the IHC test for NUT, these two cases were scored as false negatives.

Accuracy of NUT Immunohistochemistry

Of 919 non-germ cell malignancies there were 4 false negatives, and 0 false positives (Table 5). In two of the false negatives, as mentioned above, there was weak nuclear staining, which led to discrepant interpretations by the two pathologists. One of these cases was an autopsy, and therefore the weak staining may have been the result of post-mortem antigen degradation.

Of the three other false negative cases, two harbored *NUT*-variant translocations, based on FISH analyses that revealed rearrangement of *NUT* and intact *BRD4* and *BRD3* loci (data not shown). It is possible that these variant fusion proteins are expressed at lower levels than BRD3-NUT and BRD4-NUT fusion proteins, limiting detection by the IHC test described here. A precedent for this is found in the recently described *EML4-ALK* rearrangements in non-small cell lung carcinoma(7) which are not detected using antibodies and staining conditions that are otherwise quite sensitive for detecting ALK fusion proteins in anaplastic large cell lymphoma (unpublished data). Nevertheless, 2 BRD3-NUT, and 5 NUT-variant tumors with unknown partner genes did stain positively with the C52 antibody, and thus we cannot exclude (as with the autopsy case) poor tissue preservation as a contributing factor to these other false negative results.

Amongst normal tissues, cytoplasmic reactivity was seen in hepatocytes and rare renal tubular cells (see above). Weak nuclear and cytoplasmic immunoreactivity of C52 in oocytes was noted (not shown). Given that NUT is highly expressed in the germ cells of the testis, this reactivity may be due to lower level expression of endogenous NUT in oocytes. *NUT* mRNA was not detected in extracts of ovary by Northern blot(4), but it is possible that low-level *NUT* expression confined to oocytes was missed by this analysis.

Overall, C52 IHC had a sensitivity of 87% and a specificity of 100% (Table 6) for the diagnosis of NMCs amongst non-germ cell tumors. Of interest, two cases with strong nuclear reactivity (Fig. 2C) were negative for *NUT* rearrangement in our standard FISH assay and were initially scored as false positives. However, in both of these cases further FISH studies using a probe that spans *NUT* (rather than two flanking probes) revealed a break within the probe and joining of one portion with a *BRD4* centromeric probe (Fig. 2D and data not shown), consistent with the presence of cryptic *BRD4-NUT* rearrangements. Presumably the mechanism of these rearrangements, heretofore not described, involves two breaks in the DNA immediately flanking the 5' and 3' ends of *NUT*. These may permit *NUT* to be inserted into *BRD4*, while leaving a small interstitial deletion on chromosome 15 that does not affect the regions that are homologous to the split-apart *NUT* probes used in our FISH assay.

As a result of these additional studies, these two cases were reclassified as FISH false negatives, which reduced the diagnostic sensitivity of our "gold standard" FISH assay to 93%. Overall, a diagnostic sensitivity of 100% was only achieved through the combination of FISH and C52 IHC testing.

Staining of Germ Cell Tumors with NUT Antibody

Some germ cell tumors, particularly dysgerminomas (64%) and to a lesser extent seminomas and embryonal carcinomas, revealed weak, focal nuclear immunoreactivity when stained with C52 (Fig. 3A, Table 7). The staining in dysgerminomas is presumed to be due to expression of normal NUT, based on the lack of *NUT* rearrangements (n=9), and detection of *NUT* mRNA by RT-PCR (Fig. 3B). The findings are consistent with the known expression of NUT within germ cells of testis (Fig. 1B, (4)) and the immunoreactivity of oocytes.

DISCUSSION

Because NMC is a newly recognized disease, having first been defined in 2004(5), it is not widely recognized and frequently misdiagnosed as poorly differentiated carcinoma, squamous cell carcinoma, Ewing sarcoma, sinonasal undifferentiated carcinoma (SNUC), thymic carcinoma, or even neuroblastoma (Table 4). Proper diagnosis of NMCs is likely to be important, as these tumors have an unusual propensity for early, widespread hematogenous spread, and there is accumulating evidence that NMCs respond to therapeutic regimens different than those used to treat other carcinomas. The findings reported here indicate that

routine IHC with the C52 antibody can be a useful tool in diagnosing NMC. IHC with the C52 antibody has a very high predictive value and appears to be an excellent first line test for the diagnosis of NMC. The principal limitation with C52 IHC appears to be false negative results. The basis for these is uncertain at present, but may well involve technical issues such as tissue handling, fixation, and processing. For this reason, we believe that FISH for *NUT* rearrangements should be performed when C52 IHC is negative and NMC remains high on the list of differential diagnoses.

Another potential diagnostic concern raised by our study is immunoreactivity of germ cell tumors with the C52 antibody, but this is unlikely to cause confusion in practice. Many (but not all) NMCs exhibit focal squamous differentiation and can be easily distinguished from germ cell tumors on immunohistochemical grounds, as NMCs do not express germ cell markers (2). Further, germ cell tumors display only a focal (< 5% of nuclei), smooth pattern of nuclear staining (Fig. 3A), whereas NMCs display a diffuse (≥50%), speckled pattern of nuclear staining (Fig. 2B–C). With these relatively minor caveats, our data suggest that the C52 antibody is an excellent diagnostic reagent for the identification of NMC among squamous and poorly differentiated carcinomas.

Another finding is that none of the 327 predominantly smoking-related, head and neck squamous cell carcinomas analyzed by FISH revealed *NUT* rearrangement, and similarly that none of 438 such tumors stained positively with the C52 antibody. This finding reinforces the idea that NMCs have a pathogenesis distinct from that of squamous cell carcinomas arising from environmental exposures, such as smoking. Although NMC may still arise incidentally in smokers, a smoking history appears to make the diagnosis of NMC of the aerodigestive tract highly unlikely.

Acknowledgments

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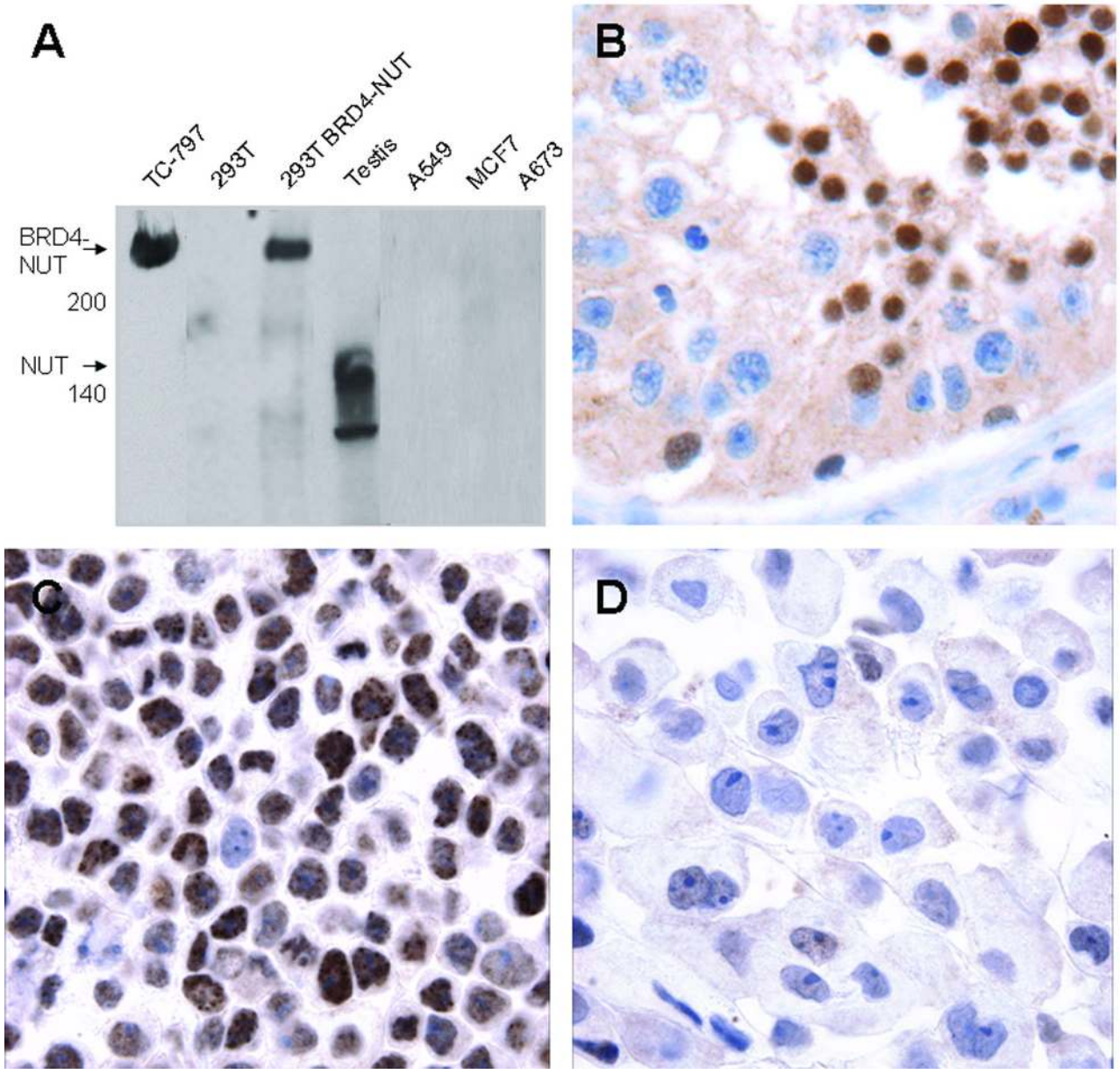

FIGURE 1.

Validation of the anti-NUT C52 monoclonal antibody by immunoblot (A) and immunohistochemistry (B–D, 400×). A, bands for BRD4-NUT (~240kDa) are seen in the NUT NMC cell line, TC-797, and in extracts prepared from 293Tcells transiently transfected with a pcDNA3 plasmid that drives expression of BRD4-NUT. Endogenous NUT (~150kDa) is seen in a lysate prepared from testis. No bands were seen in any negative control cell lines (293T cells transfected with empty pcDNA3, A549, MCF7, and A673). B, nuclear staining for NUT is seen in spermatids and a subset of spermatogonia in human testis. C, control siRNA transfected TC-797 cells reveal speckled nuclear staining, whereas NUT siRNA transfected cells (D) demonstrate significantly reduced nuclear staining. Note that the siRNA against NUT also induces marked increases in cytoplasmic volume and nuclear size, features consistent with the induction of differentiation by withdrawal of BRD4-NUT(5).

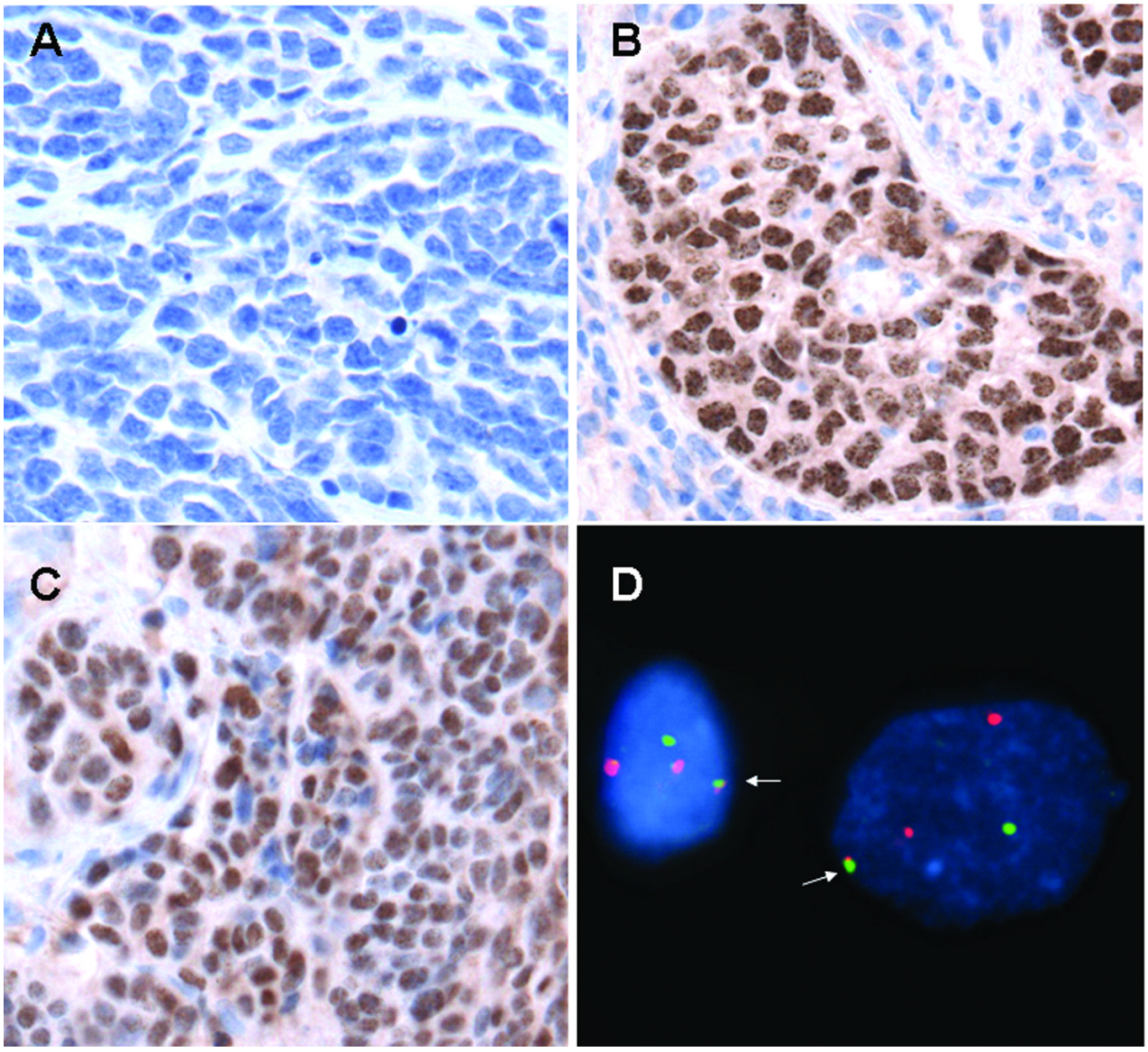

FIGURE 2.

Detection of BRD4-NUT by the C52 NUT antibody (A–C, 400×), and validated by fluorescent in situ hybridization (D, 1000×). A, typical absence of staining of a non-NMC, in this case a sinonasal undifferentiated carcinoma that was also negative for *BRD4-NUT* rearrangement by FISH (not shown). By contrast, B, known NMCs reveal diffuse (>90%), speckled, nuclear staining. In two carcinomas (one of which is shown in C), split-apart FISH was negative for *NUT* rearrangement (data not shown), but tumor cells revealed diffuse nuclear reactivity with C52. A cryptic *NUT* rearrangement (D) was subsequently demonstrated in this case (as well as the second, not shown) using a probe (red) that spans *NUT*. This probe splits and joins (arrows) the *BRD4* centromeric probe (green), consistent with the presence of a cryptic *BRD4-NUT* fusion gene.

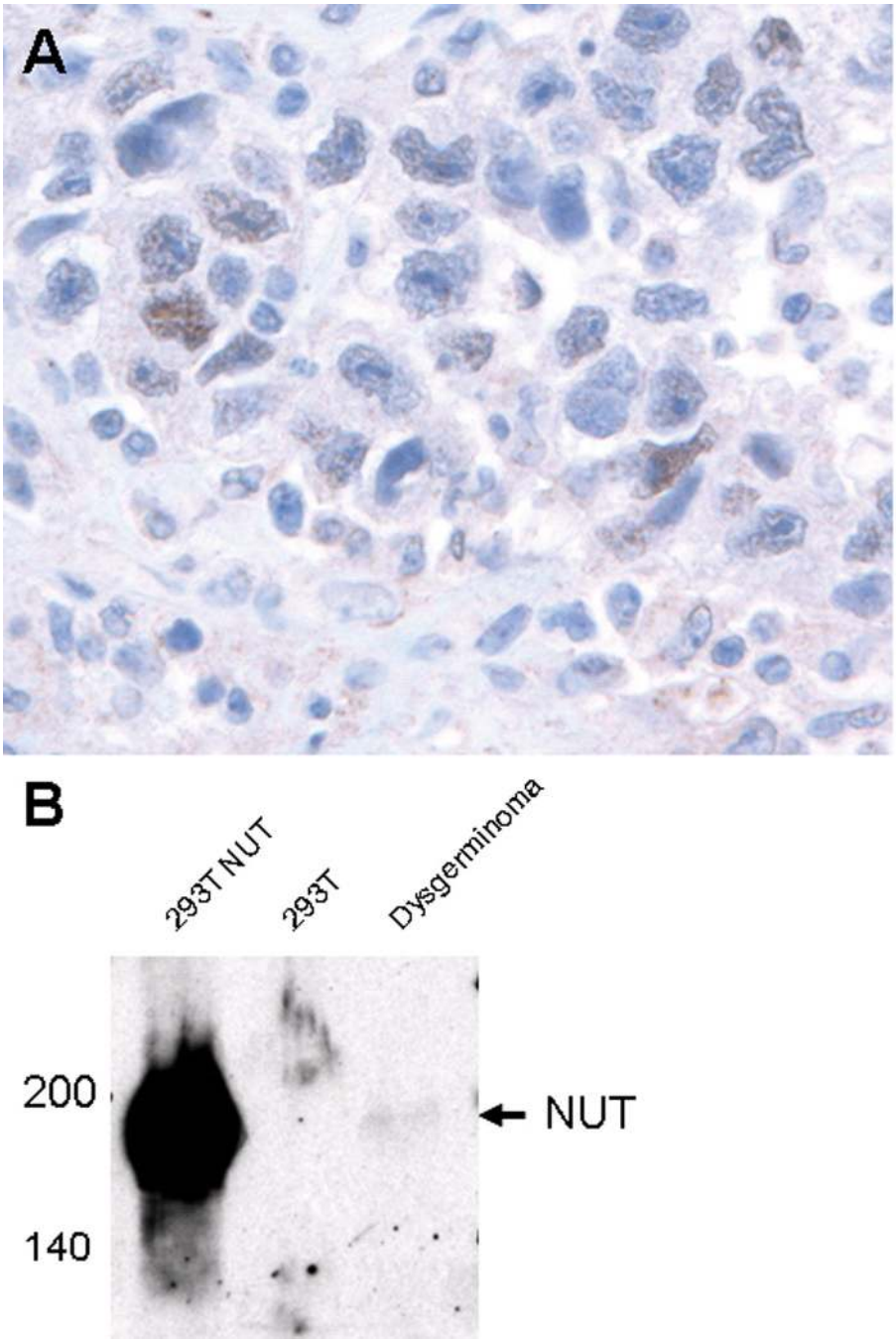

FIGURE 3.

Focal, weak nuclear staining by C52 (<5% cells) in a case of dysgerminoma (A, 400×). The staining, by contrast with that in NMCs, is smooth and not speckled. Western blot (B), stained with C52 antibody, confirms low level expression of native NUT in lysate obtained from this dysgerminoma. The positive control is an extract prepared from 293Tcells transiently transfected with pcDNA3-NUT, and the negative control is 293T cells transfected with empty pcDNA3.

Table 1

Case Characteristics: Primary Site

Primary site	No.
larynx	190
oral cavity	180
lung	88
testis	71
breast	65
prostate	64
sinonasal	53
ovary	47
colon	45
lymph node	29
pharynx	25
mediastinum	24
rectum	20
salivary gland, NOS	17
esophagus	15
kidney	15
pancreas	15
uterus (endometrium)	15
bladder	14
head&neck, NOS	11
liver	11
stomach	8
nasopharynx	7
thymus	6
bile duct	6
chest wall	5
abdomen, NOS	4
bone	3
spleen	3
bronchus	2
tonsil	2
trachea	2
epiglottis	1
adrenal	1
brain	1
placenta	1
scalp	1
thyroid	1
Total	1068

Abbreviations: NOS, not otherwise specified

Table 2

Case Characteristics: Diagnosis

Diagnosis	No.
Carcinoma	**906**
squamous CA, NOS	504
adenoCA	260
SNUC	29
poorly differentiated CA, NOS	25
transitional cell CA	13
serous CA, ovary	12
small cell CA	11
non-small cell CA	9
nasopharyngeal CA	6
hepatocellular CA	6
renal cell CA, clear cell type	6
malignant neoplasm, NOS	4
mucoepidermoid CA	4
large cell undifferentiated CA	3
renal cell CA, chromophobe	3
renal cell CA, papillary type	3
thyroid papillary CA	2
sarcomatoid CA	2
thymic CA	1
atypical carcinoid, lung	1
salivary gland CA, NOS	1
squamous papilloma	1
Germ cell tumor	**110**
embryonal carcinoma	39
seminoma	33
dysgerminoma	11
teratoma, immature	9
germ cell tumor, NOS	8
struma ovarii	4
teratoma, mature	3
yolk sac tumor	2
endodermal sinus tumor	1
Other malignant tumors	**12**
Ewing's sarcoma	3
rhabdoid tumor	2
neuroblastoma	2
olfactory neuroblastoma	2
small round blue cell tumor	1
non-Hodgkin lymphoma	1
rhabdomyoscarcoma	1
Normal tissue	**40**
lung	7
colon/rectum	5
breast	4
liver	4
prostate	4
spleen	3
kidney	2
thymus	2
tonsil	2
adrenal	1
esophagus	1
pancreas	1
placenta	1
ovary	1
testis	1
uterus (endometrium)	1
Total	1068

Abbreviations: NOS, not otherwise specified; CA, carcinoma; SNUC, sinonasal undifferentiated carcinoma

Table 3

NMC Case Characteristics: Primary Site

Primary site	No.
mediastinum	7
sinonasal	6
lung	4
nasopharynx	2
lymph node	1
epiglottis	1
bone	1
abdomen, NOS	1
chest wall	1
thymus	1
bladder	1
salivary gland, NOS	1
larynx	1

Abbreviations: NMC, Nut midline carcinoma; NOS, not otherwise specified

Table 4

NMC Case Characteristics: Diagnosis

Diagnosis	No.
squamous cell CA, NOS	11
poorly differentiated CA, NOS	8
SNUC	3
malignant neoplasm, NOS	2
thymic CA	1
neuroblastoma	1
Ewing's sarcoma	1
nasopharyngeal CA	1

Abbreviations: NMC, Nut midline carcinoma; CA, carcinoma; NOS, not otherwise specified; SNUC, sinonasal undifferentiated carcinoma.

Table 5

Staining with C52 Monoclonal Antibody to NUT

	No.	C52+	C52−
NMC	30[a]	26	4
Non-NMC carcinomas[b]	889	0	889
Normal tissue[c]	38	5[d]	33

[a] Includes two cases with cryptic *NUT* rearrangements later detected using refined FISH.

[b] 9 of these cancers were of non-epithelial origin

[c] Testis and ovary not included.

[d] cytoplasmic and nuclear reactivity (liver n = 4, renal tubules n = 1)

Table 6

Accuracy of C52 Antibody in Diagnosis of NMC

	% Sensitivity	% specificity	% PPV	% NPV
C52 IHC	87	100[a]	100	99
FISH	93	100	100	~100
FISH + C52 IHC	100	100	100	100

[a] Based on a total of 481 cases that were FISHd for *NUT* rearrangement.

Abbreviation: NMC, NUT midline carcinoma. IHC, Immunohistochemistry. FISH, Fluorescent in situ hybridization. PPV, Positive predictive value. NPV, Negative predictive value. TP, true positive. TN, true negative. FN, false negative. FP, false positive.

Sensitivity = TP/(TP + FN)

Specificity = TN/(FP + TN)

PPV = TP/(TP + FP)

NPV = TN/(TN + FN)

Table 7

C52 Staining in Germ Cell Tumors

Diagnosis	No.	C52+	C52−	% C52+NUTr[a]
dysgerminoma	11	7	4	640/9
embryonal carcinoma	35	3	32	8.60/3
endodermal sinus tumor	1		1	0
germ cell tumor, NOS	3		3	0
seminoma	33	2	31	60/1
struma ovarii	4		4	0
teratoma, immature	9		9	0
teratoma, mature	3		3	0
yolk sac tumor	2		2	0
Total	101	12	89	12

Abbreviations: NOS, not otherwise specified. NUTr, NUT rearrangement as determined by FISH.

[a]No. of evaluable cases with C52 immunoreactivity.