processed after obtaining the patient informed consent to participate in this study.

Immunostaining

For immunohistochemical analysis, TMA blocks were prepared and then deparaffinized, rehydrated, and boiled for 30 min in Tris-EDTA buffer (pH 9.0) for CDC6 or 10 mM sodium citrate buffer (pH 6.0) for cyclin D1 and YB-1 as antigen retrieval (Igarashi *et al.* 1994; Sugimura 2008). Endogenous peroxidase activity was blocked by incubation in a hydrogen peroxide solution for 30 min. The sections were then incubated with a rabbit anti-YB-1 polyclonal antibody (Abcam, 1 : 7500), a rabbit anti-cyclin D1 monoclonal antibody (Nichirei, Tokyo, Japan, 1 : 25) and a rabbit anti-CDC6 polyclonal antibody (ProteinTech Group Inc., 1 : 100). The antigen-antibody complex was visualized using Histofine Simple Stain Max Po (Multi; Nichirei, Tokyo, Japan) and 3, 3′-diaminobenzidine tetrahydrochloride. Counterstaining was carried out using hematoxylin. For the evaluation of YB-1, cyclin D1, and CDC6 expression, the number of stained cells was counted, and at least five high-power fields were chosen randomly for scoring of the percentage of cells with positive staining among 1000 cells examined per section.

Expression of YB-1, cyclin D1, and CDC6 was classified into three categories: score 0, no staining at all or expression in <10% of cancer cells; score 1+, expression >10% and <50%; score 2+, expression >50%. The expressions of YB-1, cyclin D1, and CDC6 were defined as follows: scores 1+ and 2+ were regarded positive, and score 0 was regarded negative (Fig. S1 in Supporting Information).

Statistical analysis

Data are presented as mean ± SD. Data were analyzed by Student's *t*-test or Fisher's exact test, where $P < 0.05$ was considered to be statistically significant. All data analysis was carried out using a statistical software package JMP® software (version 10, SAS Institute Inc., Cary, NC, USA).

Acknowledgements

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References

Bakiri, L., Lallemand, D., Bossy-Wetzel, E. & Yaniv, M. (2000) Cell cycle-dependent variations in c-Jun and JunB phosphorylation: a role in the control of cyclin D1 expression. *EMBO J.* **19**, 2056–2068.

Basaki, Y., Taguchi, K.-I., Izumi, H., Murakami, Y., Kubo, T., Hosoi, F., Watari, K., Nakano, K., Kawaguchi, H., Ohno, S., Kohno, K., Ono, M. & Kuwano, M. (2010) Y-box binding protein-1 (YB-1) promotes cell cycle progression through CDC6-dependent pathway in human cancer cells. *Eur. J. Cancer* **46**, 954–965.

Boulon, S., Dantonel, J.-C., Binet, V., Vié, A., Blanchard, J.-M., Hipskind, R.A. & Philips, A. (2002) Oct-1 potentiates CREB-driven cyclin D1 promoter activation via a phospho-CREB- and CREB binding protein-independent mechanism. *Mol. Cell. Biol.* **22**, 7769–7779.

Ding, L., Getz, G., Wheeler, D.A., *et al.* (2008) Somatic mutations affect key pathways in lung adenocarcinoma. *Nature* **455**, 1069–1075.

Dolfini, D. & Mantovani, R. (2012) YB-1 (YBX1) does not bind to Y/CCAAT boxes in vivo. *Oncogene* **19**, 521.

Eliseeva, I.A., Kim, E.R., Guryanov, S.G., Ovchinnikov, L.P. & Lyabin, D.N. (2011) Y-box-binding protein 1 (YB-1) and its functions. *Biochemistry Mosc.* **76**, 1402–1433.

Gil, J. & Peters, G. (2006) Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. *Nat. Rev. Mol. Cell Biol.* **7**, 667–677.

Gonzalez, S., Klatt, P., Delgado, S., Conde, E., Lopez-Rios, F., Sanchez-Cespedes, M., Mendez, J., Antequera, F. & Serrano, M. (2006) Oncogenic activity of Cdc6 through repression of the INK4/ARF locus. *Nature* **440**, 702–706.

Guttridge, D.C., Albanese, C., Reuther, J.Y., Pestell, R.G. & Baldwin, A.S. (1999) NF-κB controls cell growth and differentiation through transcriptional regulation of cyclin D1. *Mol. Cell. Biol.* **19**, 5785–5799.

Homer, C., Knight, D.A., Hananeia, L., Sheard, P., Risk, J., Lasham, A., Royds, J.A. & Braithwaite, A.W. (2005) Y-box factor YB1 controls p53 apoptotic function. *Oncogene* **24**, 8314–8325.

Igarashi, H., Sugimura, H., Maruyama, K., Kitayama, Y., Ohta, I., Suzuki, M., Tanaka, M., Dobashi, Y. & Kino, I. (1994) Alteration of immunoreactivity by hydrated autoclaving, microwave treatment, and simple heating of paraffin-embedded tissue sections. *APMIS* **102**, 295–307.

Imada, K., Shiota, M., Kohashi, K., Kuroiwa, K., Song, Y., Sugimoto, M., Naito, S. & Oda, Y. (2013) Mutual regulation between Raf/MEK/ERK signaling and Y-Box-binding protein-1 promotes prostate cancer progression. *Clin. Cancer Res.* **7**, 7.

Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E. & Forman, D. (2011) Global cancer statistics. *CA Cancer J. Clin.* **61**, 69–90.

Jurchott, K., Bergmann, S., Stein, U., Walther, W., Janz, M., Manni, I., Piaggio, G., Fietze, E., Dietel, M. & Royer, H.D. (2003) YB-1 as a cell cycle-regulated transcription factor facilitating cyclin A and cyclin B1 gene expression. *J. Biol. Chem.* **278**, 27988–27996.

Kanie, T., Onoyama, I., Matsumoto, A., Yamada, M., Nakatsumi, H., Tateishi, Y., Yamamura, S., Tsunematsu, R., Matsumoto, M. & Nakayama, K.I. (2012) Genetic reevaluation of the role of F-box proteins in cyclin D1 degradation. *Mol. Cell. Biol.* **32**, 590–605.

Kim, J.K. & Diehl, J.A. (2009) Nuclear cyclin D1: an oncogenic driver in human cancer. *J. Cell. Physiol.* **220**, 292–296.

Klein, E.A. & Assoian, R.K. (2008) Transcriptional regulation of the cyclin D1 gene at a glance. *J. Cell Sci.* **121**, 3853–3857.

Kohno, K., Izumi, H., Uchiumi, T., Ashizuka, M. & Kuwano, M. (2003) The pleiotropic functions of the Y-box-binding protein, YB-1. *BioEssays* **25**, 691–698.

Kotake, Y., Ozawa, Y., Harada, M., Kitagawa, K., Niida, H., Morita, Y., Tanaka, K., Suda, T. & Kitagawa, M. (2013) YB1 binds to and represses the p16 tumor suppressor gene. *Genes Cells* **18**, 999–1006.

Lasham, A., Print, C.G., Woolley, A.G., Dunn, S.E. & Braithwaite, A.W. (2013) YB-1: oncoprotein, prognostic marker and therapeutic target? *Biochem. J.* **449**, 11–23.

Lasham, A., Samuel, W., Cao, H., Patel, R., Mehta, R., Stern, J.L., Reid, G., Woolley, A.G., Miller, L.D., Black, M.A., Shelling, A.N., Print, C.G. & Braithwaite, A.W. (2012) YB-1, the E2F pathway, and regulation of tumor cell growth. *J. Natl Cancer Inst.* **104**, 133–146.

Lee, R.J., Albanese, C., Stenger, R.J., Watanabe, G., Inghirami, G., Haines, G.K., Webster, M., Muller, W.J., Brugge, J.S., Davis, R.J. & Pestell, R.G. (1999) pp60v-src induction of cyclin D1 requires collaborative interactions between the extracellular signal-regulated kinase, p38, and Jun kinase pathways: a role for cAMP response element-binding protein and activating transcription factor-2 In pp60v-src signaling in breast cancer cells. *J. Biol. Chem.* **274**, 7341–7350.

Marchetti, A., Felicioni, L., Malatesta, S., Grazia Sciarrotta, M., Guetti, L., Chella, A., Viola, P., Pullara, C., Mucilli, F. & Buttitta, F. (2011) Clinical features and outcome of patients with non-small-cell lung cancer harboring BRAF mutations. *J. Clin. Oncol.* **29**, 3574–3579.

Molina, J.R., Yang, P., Cassivi, S.D., Schild, S.E. & Adjei, A.A. (2008) Non-small cell lung cancer: epidemiology, risk factors, treatment, and survivorship. *Mayo Clin. Proc.* **83**, 584–594.

Musgrove, E.A., Caldon, C.E., Barraclough, J., Stone, A. & Sutherland, R.L. (2011) Cyclin D as a therapeutic target in cancer. *Nat. Rev. Cancer* **11**, 558–572.

Nagata, D., Suzuki, E., Nishimatsu, H., Satonaka, H., Goto, A., Omata, M. & Hirata, Y. (2001) Transcriptional activation of the cyclin D1 gene is mediated by multiple cis-elements, including SP1 sites and a cAMP-responsive element in vascular endothelial cells. *J. Biol. Chem.* **276**, 662–669.

Okamoto, T., Izumi, H., Imamura, T., Takano, H., Ise, T., Uchiumi, T., Kuwano, M. & Kohno, K. (2000) Direct interaction of p53 with the Y-box binding protein, YB-1: a mechanism for regulation of human gene expression. *Oncogene* **19**, 6194–6202.

Okudela, K., Suzuki, M., Kageyama, S., *et al.* (2007) PIK3CA mutation and amplification in human lung cancer. *Pathol. Int.* **57**, 664–671.

Oxnard, G.R., Binder, A. & Janne, P.A. (2013) New targetable oncogenes in non-small-cell lung cancer. *J. Clin. Oncol.* **31**, 1097–1104.

Petrakis, T.G., Vougas, K. & Gorgoulis, V.G. (2012) Cdc6: a multi-functional molecular switch with critical role in carcinogenesis. *Transcription* **3**, 124–129.

Sacco, E., Hasan, M.M., Alberghina, L. & Vanoni, M. (2012) Comparative analysis of the molecular mechanisms controlling the initiation of chromosomal DNA replication in yeast and in mammalian cells. *Biotechnol. Adv.* **30**, 73–98.

Sanders, H.R. & Albitar, M. (2010) Somatic mutations of signaling genes in non-small-cell lung cancer. *Cancer Genet. Cytogenet.* **203**, 7–15.

Sengupta, S., Mantha, A.K., Mitra, S. & Bhakat, K.K. (2011) Human AP endonuclease (APE1/Ref-1) and its acetylation regulate YB-1-p300 recruitment and RNA polymerase II loading in the drug-induced activation of multidrug resistance gene MDR1. *Oncogene* **30**, 482–493.

Shi, J.-H., Zheng, B., Chen, S., Ma, G.-Y. & Wen, J.-K. (2012) Retinoic acid receptor α mediates all-trans-retinoic acid-induced Klf4 gene expression by regulating Klf4 promoter activity in vascular smooth muscle cells. *J. Biol. Chem.* **287**, 10799–10811.

Shiota, M., Izumi, H., Onitsuka, T., Miyamoto, N., Kashiwagi, E., Kidani, A., Yokomizo, A., Naito, S. & Kohno, K. (2008) Twist promotes tumor cell growth through YB-1 expression. *Cancer Res.* **68**, 98–105.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R. & Ben-Ze'ev, A. (1999) The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. *Proc. Natl Acad. Sci. USA* **96**, 5522–5527.

Singhal, S., Vachani, A., Antin-Ozerkis, D., Kaiser, L.R. & Albelda, S.M. (2005) Prognostic implications of cell cycle, apoptosis, and angiogenesis biomarkers in non-small cell lung cancer: a review. *Clin. Cancer Res.* **11**, 3974–3986.

Sugimura, H. (2008) Detection of chromosome changes in pathology archives: an application of microwave-assisted fluorescence in situ hybridization to human carcinogenesis studies. *Carcinogenesis* **29**, 681–687.

Sun, Y. (2006) p53 and its downstream proteins as molecular targets of cancer. *Mol. Carcinog.* **45**, 409–415.

Witzel, I.I., Koh, L.F. & Perkins, N.D. (2010) Regulation of cyclin D1 gene expression. *Biochem. Soc. Trans.* **38**, 217–222.

Wu, J., Lee, C., Yokom, D., Jiang, H., Cheang, M.C.U., Yorida, E., Turbin, D., Berquin, I.M., Mertens, P.R., Iftner, T., Gilks, C.B. & Dunn, S.E. (2006) Disruption of the Y-box binding protein-1 results in suppression of the epidermal growth factor receptor and HER-2. *Cancer Res.* **66**, 4872–4879.

Yokoyama, H., Harigae, H., Takahashi, S., Furuyama, K., Kaku, M., Yamamoto, M. & Sasaki, T. (2003) Regulation

of YB-1 gene expression by GATA transcription factors. *Biochem. Biophys. Res. Commun.* **303**, 140–145.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1 Representative samples of positive and negative findings of YB-1, cyclin D1, and CDC6.

Figure S2 Effect of YB-1 knockdown on cell proliferation and cell cycle in non-small-cell lung cancer cells.

Figure S3 Effect of YB-1 knockdown on expression of cyclin D1 protein and mRNA in non-small-cell lung cancer cells.

Table S1 Patient characteristics of NSCLCs

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Teaching cases

A rare Japanese case with a NUT midline carcinoma in the nasal cavity: A case report with immunohistochemical and genetic analyses

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ABSTRACT

Background: NUT (nuclear protein in testis) midline carcinoma (NMC) is a recently described aggressive malignancy that is genetically defined by rearrangements of the *NUT* locus at 15q14. In approximately two-thirds of cases, the characteristic t(15;19) results in the fusion oncogene *BRD4-NUT*. Only 10 sinonasal NMCs have been documented, none of which were Japanese cases.

Case presentation: An 18-year-old woman was admitted because of a rapidly progressing tumor in the nasal cavity. A biopsy revealed an undifferentiated neoplasm without squamous differentiation. The tumor cells had round to oval nuclei with vesicular chromatin, prominent nucleoli, and scant cytoplasm. Immunohistochemical staining demonstrated a strong positivity for vimentin and NUT, with focal CD138 and only spotty EMA and cytokeratin AE1/AE3 staining. Cytogenetic and fluorescence in situ hybridization analyses revealed a t(15;19) and *BRD4-NUT* gene rearrangement. Direct sequencing identified the in-frame fusion of exon11 of *BRD4* with exon2 of *NUT*. The patient was transferred to another hospital for chemoradiotherapy.

Conclusion: We herein describe the first Japanese case with an NMC of the sinonasal cavity, providing detailed and unambiguous cyto- and molecular genetic information on *BRD4-NUT*-rearrangement. The accumulation of cases with well-documented genetic data should provide clues to the treatment of this tumor entity.

Introduction

Several malignant tumors of the sinonasal tract may present with an undifferentiated morphology [6,20]. Overall, these tumors pose significant diagnostic challenges to attending surgical pathologists, especially in cases in which a limited amount of biopsy material is available [6] and in cases that do not exhibit the typical immunohistochemical results [3]. Among these sinonasal malignant neoplasms, nuclear protein in testis (NUT) midline carcinoma (NMC) is a recently recognized entity that is characterized by a poor prognosis [1,20].

The current recognition of the NMC entity is genetically defined by rearrangements of the *NUT* locus at 15q14, resulting in a fusion transcript with a member of the bromodomain-containing protein (BRD) family, usually *BRD4* located on chromosome 19p13.1 [7,10]. As NMC is a recently described tumor entity, it is still unfamiliar to most pathologists [14,16,20]. The histological features of tumors that have been reported as NMCs range from entirely undifferentiated carcinomas to carcinomas with prominent squamous differentiation [2–4,10,19]. Thus, the diagnosis of NMC simply based on morphology is difficult. Until recently, only one molecular method within the realm of diagnostic pathology, i.e., fluorescence in situ hybridization (FISH), was available for the robust demonstration of a rearrangement of the *NUT* gene [3,8,19]. Consequently, this entity has been commonly undiagnosed or misdiagnosed in clinical pathology practice, and comprehensive information, such as the correlation between the molecular features and the biological behaviors of this cancer, is limited. Since the identification of a somatic rearrangement involving the *NUT* gene in NMC [7], only

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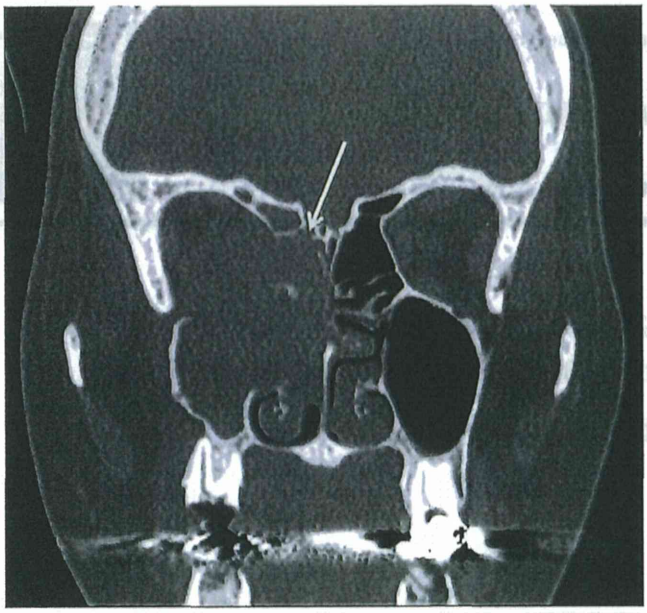

Fig. 1. CT scan of head obtained upon admission. The tumor occupies the right nasal cavity and maxillary and ethmoidal sinus, involving the ethmoid bone (arrow).

a few reports have determined the fusion gene in clinical specimens using a direct sequencing method [7,9,12,23]. NMCs are rare [1,10,20], and the geographic distribution of reported cases has been concentrated in the United States [1]. NMCs most often occur in the midline, including the head and neck, and the thorax [10,20]. Although the sinonasal tract is considered a preferential site in the head and neck region, only 10 sinonasal NMCs have been documented [2,3,8,13,19], none of which were Japanese cases.

In this article, we describe a new Japanese case with NMC in the nasal cavity; the NMC was diagnosed using immunohistochemistry with a highly sensitive and specific NUT monoclonal antibody [11]. In addition, a *BRD4-NUT* fusion gene, as the mechanism of NUT overexpression, was defined by karyotyping as well as molecular methods, including FISH and RT-PCR direct sequencing.

Clinical summary

An 18-year-old woman sought medical advice because of nasal discharge containing blood accompanied by pain with a 1-month duration. A computed tomography scan performed at the time of hospitalization revealed a mass in the right nasal cavity, and maxillary and ethmoidal sinus (Fig. 1). The mass involved the ethmoid bone (Fig. 1, arrow). A biopsy was performed.

Material and methods

Histopathologic and immunohistochemical examination

All the tissues were fixed in 10% buffered formalin and were embedded in paraffin after routine processing, followed by sectioning and staining with hematoxylin and eosin (H&E). Immunostaining was performed using antibodies for the following antigens: CD34 class II (DAKO, Glostrup, Denmark), CD45 (DAKO), CD56 (Leica Biosystems, Newcastle, United Kingdom), CD99 (DAKO), CD138/Syndecan-1 (DAKO), c-kit (DAKO), cytokeratin AE1/AE3 (DAKO), desmin (DAKO), epithelial membrane antigen (EMA, DAKO), hCG (DAKO), myogenin (DAKO), neuron-specific enolase (Nichirei, Tokyo, Japan), NUT (Cell Signaling Technologies Inc., Danvers, MA), PLAP (Leica Biosystems), S-100 (DAKO), and synaptophysin (DAKO).

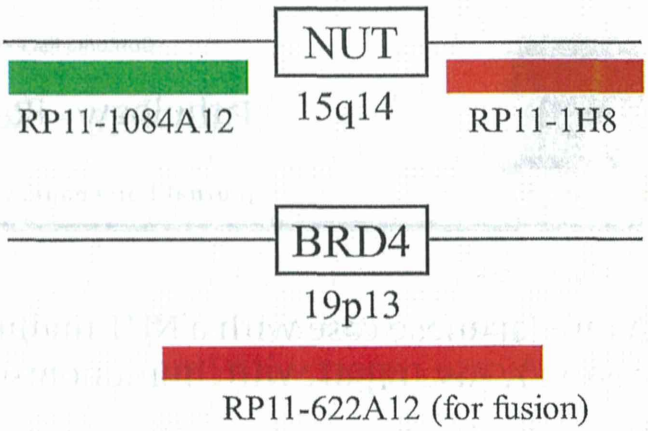

Fig. 2. Chromosomal positions of FISH probes flanking or including the genes of interest (*NUT* and *BRD4*).

FISH

The FISH procedure was performed as previously reported [21,22]. FISH probes were prepared from the Bacterial Artificial Chromosome (BAC) library. BAC probes flanking *NUT* (RP11-1084A12 and RP11-1H8) and a BAC probe containing *BRD4* (RP11-622A12) were purchased from Advanced GenoTechs Co. (Tsukuba, Japan) (Fig. 2). All the probes were confirmed to be correct by hybridizing them to a metaphase human chromosome spread (data not shown). To detect the break apart of NUT, the RP11-1084A12 probe was nick-translated using Green dUTP (2N32-50; Abbot, IL, USA) and the RP11-1H8 probe was nick-translated using Orange dUTP (2N33-50; Abbot). The combinations of RP11-1084A12, RP11-1H8, and RP11-622A12 were used to detect the fusion of *BRD4* and *NUT*. To detect the fusion, the RP11-622A12 probe was nick-translated using Orange dUTP (2N33-50), while both the RP11-1084A12 and the RP11-1H8 probes were nick-translated using Green dUTP (2N32-50). The nuclei were stained with 4,6-diamino-2-phenyl indole dihydrochloride (DAPI, Abbot). The slides were reviewed manually with at least 50 tumor nuclei evaluated for each case, and a cutoff of >30% nuclei showing a split or fused signal was considered positive for the rearrangement of the genes.

Direct sequencing

BRD4-NUT fusion cDNA was amplified using PCR with Phusion (New England BioLabs, Beverly, MA, USA); direct sequencing was then performed as described previously [7]. The primers BR2276F (AAGTTGATGTGATTGCCGGCTCCTC) and *NUT*1194R (GAGGTCTCTGGGCTTTACGCTGACG) were used [7]. Gel-purified PCR products were cycle-sequenced by the incorporation of ABI PRISM Big Dye Terminators (Perkin-Elmer, Inc., Wellesley, MA) and analyzed using an ABI 3130 sequencer (Life Science Technologies, Carlsbad, CA, USA).

Pathological findings

The small amount of biopsy material that was obtained revealed an undifferentiated neoplasm with necrosis (Fig. 3a). The tumor cells had round to oval nuclei with vesicular chromatin, prominent nucleoli, and scant cytoplasm (Fig. 3b). Frequent mitotic figures (10/5 hpf) were observed (Fig. 3b, arrow). Squamous differentiation was not identified.

Immunohistochemical staining demonstrated a strong vimentin positivity (Fig. 4a) with focal CD138 (Fig. 4b) and only spotty EMA (Fig. 4c) and cytokeratin AE1/AE3 positivity. Negative staining

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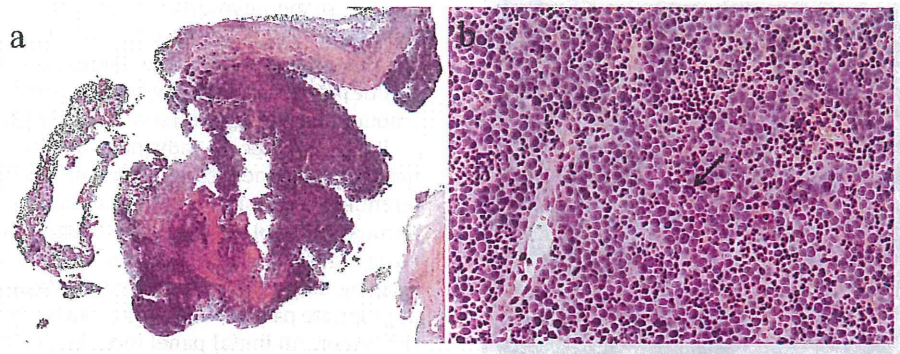

Fig. 3. Representative histologic images. (a) Sheets of undifferentiated malignant cells with focal necrosis are visible. (b) The tumor cells had round to oval nuclei with vesicular chromatin, prominent nucleoli, and scant cytoplasm. Frequent mitotic figures are visible (arrow).

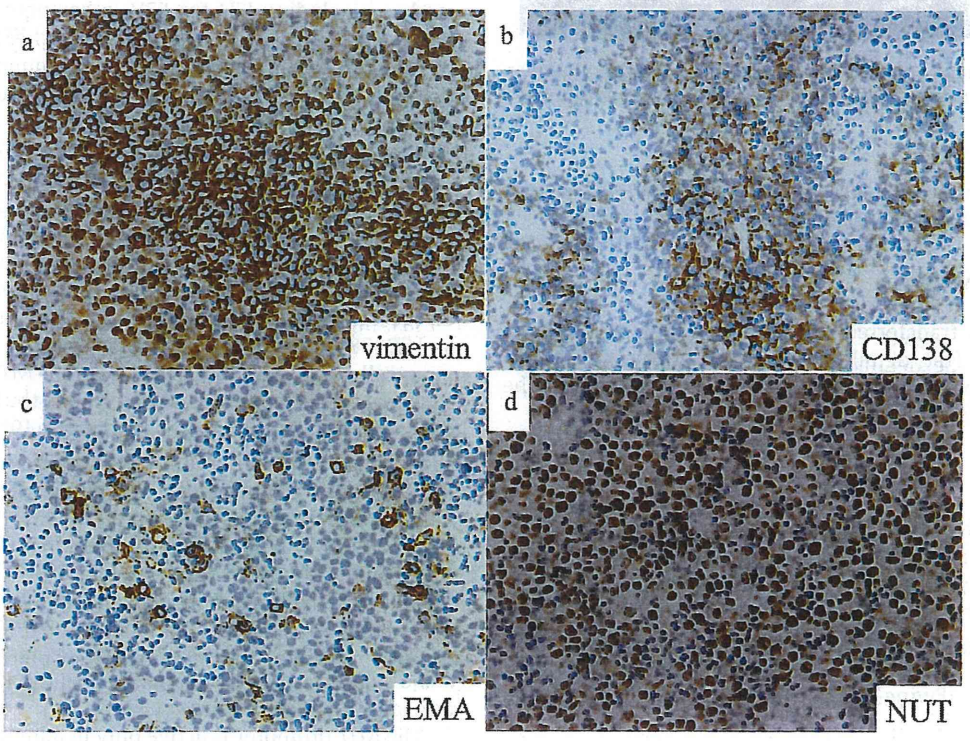

Fig. 4. Immunohistochemical findings of the NUT midline carcinoma. (a) Tumor cells show strong vimentin immunoreactivity. (b) The tumor cells are focally positive for CD138. (c) Positive staining for EMA was observed in a few tumor cells. (d) Immunohistochemistry shows a nuclear staining pattern for NUT.

results were obtained for S-100, CD34, CD99, neuron-specific enolase, CD56, synaptophysin, myogenin, desmin, PLAP, c-kit, hCG, and CD45. A diagnosis of "malignant tumor with necrosis" was made. Because of the strong vimentin positivity and focal positivity for CD138, additional immunohistochemical analyses, in situ hybridization (ISH), and a chromosome analysis were performed to enable a differential diagnosis of poorly differentiated carcinoma and plasmacytoma. ISH showed no staining for Epstein–Barr virus (EBV)-encoded RNA or the kappa and lambda light chains of immunoglobulins. These findings suggested a diagnosis of poorly differentiated carcinoma, rather than plasmacytoma. In addition, immunohistochemistry showed a nuclear staining pattern for NUT (Fig. 4d) and focal positivity for p63. A chromosome analysis identified a 46, XX, t(15;19)(q14;p13) pattern in all 9 cells (Fig. 5). These results strongly suggested a NUT midline carcinoma.

Next, FISH and RT-PCR were performed to confirm the rearrangement of *BRD4* and *NUT*. A FISH analysis revealed the break apart of *NUT* and the fusion of *BRD4* and *NUT* (Fig. 6). Direct sequencing of the 1152-bp fragments (Fig. 7a) showed that nt 2380 of *BRD4* (accession number AF386649) was fused with nt 172 of *NUT*

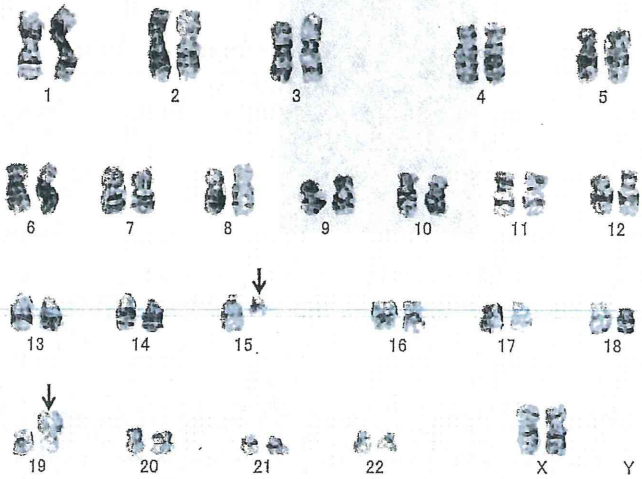

Fig. 5. Karyotype demonstrating the t(15;19)(q14;p13). The derivative chromosomes formed from the translocation are indicated by the arrows.

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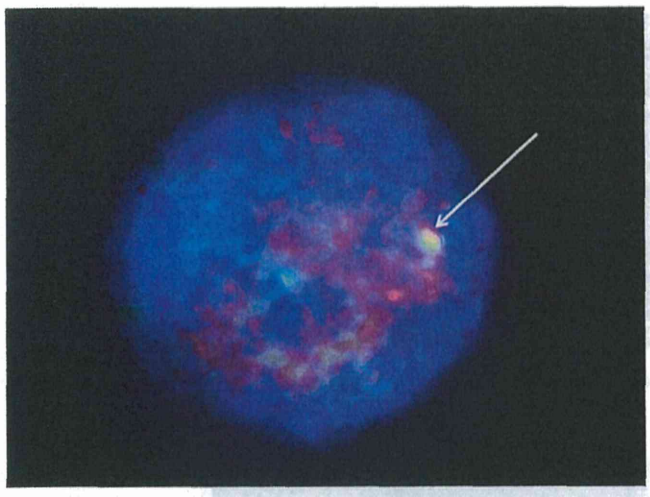

Fig. 6. Representative finding of FISH. A dual-color FISH analysis shows the fusion gene as a single yellow (overlapping) signal (arrow), including an orange (*BRD4*) and green (*NUT*) signal.

(accession number AF482429) in frame (Fig. 7b). These results confirmed the rearrangement of *BRD4* and *NUT*.

Treatment and follow up

The patient was transferred to another hospital to undergo chemoradiotherapy (the details of which are unknown), which resulted in the almost complete disappearance of the tumor. The patient is alive and well at 12 months after her diagnosis.

Discussion

The histologic features of NMC are, unfortunately, not pathognomonic, since the morphology is that of a poorly differentiated carcinoma with or without squamous differentiation [2–4,10,19]. Several malignant tumors occurring in the sinonasal tract may exhibit an undifferentiated morphology [3,6,20]. The differential diagnosis of these tumors includes hemato-lymphoid malignancies, melanomas, Ewing sarcoma/primitive neuroectodermal tumors, rhabdomyosarcoma, olfactory neuroblastoma, and germ cell tumors as well as poorly differentiated carcinomas, including NMC [3,6,20]. Overall, these lesions pose significant diagnostic difficulties for the surgical pathologist, especially when only a limited amount of biopsy material is available [3].

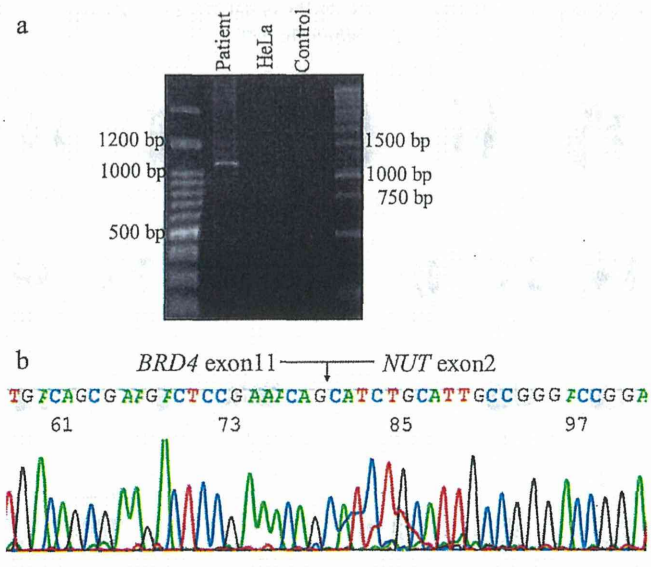

Fig. 7. Detection of BRD4-NUT. (a) PCR amplification (1152 bp) with BR2276F and *NUT*1194R primers. (b) Partial sequence chromogram showing the in-frame fusion of BRD4 exon11 with NUT exon2.

Based on recent advances in immunohistochemistry and molecular diagnostics, the current diagnostic strategy for undifferentiated tumors of the nasal cavities has been proposed [6]. Through careful microscopic examination of hematoxylin and eosin-stained sections, and in view of clinical information and imaging data, a list of differential diagnoses can be made and an appropriate panel of antibodies can be chosen to further categorize the tumor. An initial panel including cytokeratins, synaptophysin, S-100 protein, desmin, and CD45 may allow the classification of most lesions or may help to narrow the list of differential diagnoses [6]. Further refinement can be obtained through second-line markers, including ISH for EBV, other neuroendocrine markers, melanocytic markers, myogenin, CD99, other lymphocyte markers, CD138, and light chains [6]. Finally, immunohistochemistry with NUT antibodies [11] can differentiate NMC from other tumors, and molecular analysis can further assist in the recognition of a *NUT* rearrangement, which is specific for NMC [3,7–9,12,19,23].

As described above, the immunohistochemical findings in our case, including strong vimentin positivity and focal positivity for CD138, only spotty positivity for cytokeratin AE1/AE3 and EMA, and negativity for other markers, shortened the list of differential diagnoses to two: poorly differentiated carcinoma and plasmacytoma. CD138 is highly expressed by epithelial cells, as well as plasma cells, and CD138 expression is associated with the differentiation of squamous cell carcinoma [15,17,18]. The *BRD4-NUT* fusion gene encodes a protein that is thought to be involved in the blockage of epithelial differentiation and squamous maturation [9]. In addition, our case showed a poor differentiation, without squamous differentiation. Together, these results led us to expect that the expression of CD138 would be almost absent in our case. However, our case was positive for CD138, but only spotty for other epithelial markers such as AE1/AE3 and EMA. These results made a differential diagnosis more difficult, although the results encouraged us to perform immunohistochemistry using NUT antibodies, which eventually led to a diagnosis of NMC. The accumulation of more cases is needed to avoid achieving a correct diagnosis in a roundabout manner based on the histological features of NMCs.

The *NUT* promoter is active only in adult testis and ciliary ganglion [7,10,20]. As a result, only one of the two fusion genes, e.g., *BRD-NUT* (where the BRD4 promoter and the bromodomains drive the aberrant NUT expression and chromatin binding), but not *NUT-BRD*, is expressed [10]. Until recently, a definitive diagnosis of NMC was made by demonstrating a *NUT* rearrangement using dual color, split-apart FISH using probes flanking *NUT* or by demonstrating a *BRD4-NUT* fusion transcript using RT-PCR [11]. In approximately two-thirds of cases, *NUT* is fused to *BRD4*, forming the *BRD4-NUT* fusion gene [7,10]. The remaining one-third of cases are *NUT*-variants, where the partner gene is *BRD3* or some other uncharacterized gene [8,9]. Whether *NUT*-variant carcinomas differ in outcome, compared with *BRD4-NUT* carcinomas, remains controversial [1,8,10]. To demonstrate the fusion genes, FISH is preferred from a practical perspective because it is capable of detecting all NMCs, including all *NUT*-variants, whereas RT-PCR can currently only detect *BRD3*- or *BRD4-NUT* tumors [10]. Thus, information concerning the breakpoints in the *BRD* or *NUT* genes using RT-PCR direct sequencing is scarce: only four reports have successfully defined the breakpoints of the genes in clinical specimens using direct sequencing [7,9,12,23]. The breakpoints occurred within exon10 of *BRD4* [7], exon11 of *BRD4* [12,23], exon10b of *BRD4* [9], or exon9 of *BRD3* [9], and exon2 of *NUT*, fusing the business end of *BRD4*, encoding both acetyl-histone binding bromodomains and

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the extraterminal domain, with virtually the entire *NUT* gene. Our case showed that exon11 of *BRD4* was fused in-frame to exon2 of *NUT*, similar to a Japanese case with pulmonary NMC [23]. It is possible that improved identification of the breakpoints of the targeted gene might reveal an association between variations in the breakpoints and the clinicopathological features, including the location of tumors, the race of patients, the responsiveness to therapies, and the degree of squamous differentiation.

The specific NUT monoclonal antibody has been very recently established [11]. The immunohistochemical staining had a sensitivity of 87%, a specificity of 100%, a negative predictive value of 99%, and a positive predictive value of 100% [11]. The commercial circulation of this highly sensitive and specific NUT monoclonal antibody [11] has facilitated the unambiguous diagnosis of NMC, making it possible to assess the frequency and prognosis of NMC amongst a large population of patients [1,2]. One previous report showed that 3 (2%) of 151 primary sinonasal carcinomas were NMCs [2]. In this previous report, most of the patients who were younger than 50 years old and were diagnosed as having sinonasal undifferentiated carcinoma (3 out of 4, 75%) were found to actually have NMCs. Thus, the inclusion of NUT immunostaining as a part of the diagnostic workup for any sinonasal carcinoma with an undifferentiated component is recommended. Another study, the largest cohort of patients with NMC, showed that the median overall survival for patients was 6.7 months [1]. The 2-year progression-free survival (PFS) was 9%, and the 2-year overall survival (OS) was 19%. A multivariate analysis suggested that the extent of surgical resection and the initial radiotherapy were independent predictors of the PFS and OS, whereas no chemotherapeutic regimen improved the outcome [1]. Our patient underwent chemoradiotherapy (the details of which are unknown), which resulted in the almost complete disappearance of the tumor. Careful follow up is needed to evaluate the effectiveness of this treatment.

Data from the growing number of identified cases can be used to develop effective targeted therapies. One candidate therapy is a bromodomain inhibitor. A study [9] using NMC cell lines showed that the knockdown of the *NUT* fusions resulted in the dramatic differentiation and growth arrest of the malignant cells. Furthermore, the bromodomain inhibitor JQ1 was shown to be capable of displacing the *BRD4-NUT* fusion protein from chromatin, inducing a rapid differentiation and arrest of proliferation in NUT midline carcinoma cell lines [5]. In addition, JQ1 showed an excellent efficacy in murine xenograft models of NUT midline carcinoma, resulting in tumor differentiation and regression and an increase in survival [5]. The clinical translation of this research requires robust, histological outcome data, including information regarding the association between the breakpoints of the targeted genes and the sensitivity to new drugs.

In this article, we have described a new Japanese case with NMC in the nasal cavity that is pertinent to the expansion of knowledge regarding this rare tumor. Furthermore, the identification of the *BRD4-NUT* fusion gene using direct sequencing in this tumor has provided new insight as to its influence on clinicopathological features, including the location of tumors, the race of patients, the degree of squamous differentiation, and the responsiveness to therapy.

Conflict of interest

The authors have no conflicts of interest to declare.

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References

[1] D.E. Bauer, C.M. Mitchell, K.M. Strait, C.S. Lathan, E.B. Stelow, S.C. Luer, S. Muhammed, A.G. Evans, L.M. Sholl, J. Rosai, E. Giraldi, R.P. Oakley, C. Rodriguez-Galindo, W.B. London, S.E. Sallan, J.E. Bradner, C.A. French, Clinicopathologic features and long-term outcomes of NUT midline carcinoma, Clin. Cancer Res. 18 (2012) 5773–5779.
[2] J.A. Bishop, W.H. Westra, NUT midline carcinomas of the sinonasal tract, Am. J. Surg. Pathol. 36 (2012) 1216–1221.
[3] B.N. Davis, R.G. Karabakhtsian, A.L. Pettigrew, S.M. Arnold, C.A. French, Y.M. Brill, Nuclear protein in testis midline carcinomas: a lethal and underrecognized entity, Arch. Pathol. Lab. Med. 135 (2011) 1494–1498.
[4] A.G. Evans, C.A. French, M.J. Cameron, C.D. Fletcher, D.M. Jackman, C.S. Lathan, L.M. Sholl, Pathologic characteristics of NUT midline carcinoma arising in the mediastinum, Am. J. Surg. Pathol. 36 (2012) 1222–1227.
[5] P. Filippakopoulos, J. Qi, S. Picaud, Y. Shen, W.B. Smith, O. Fedorov, E.M. Morse, T. Keates, T.T. Hickman, I. Felletar, M. Philpott, S. Munro, M.R. McKeown, Y. Wang, A.L. Christie, N. West, M.J. Cameron, B. Schwartz, T.D. Heightman, N. La Thangue, C.A. French, O. Wiest, A.L. Kung, S. Knapp, J.E. Bradner, Selective inhibition of BET bromodomains, Nature 468 (2010) 1067–1073.
[6] A. Franchi, A. Palomba, A. Cardesa, Current diagnostic strategies for undifferentiated tumours of the nasal cavities and paranasal sinuses, Histopathology 59 (2011) 1034–1045.
[7] C.A. French, I. Miyoshi, I. Kubonishi, H.E. Grier, A.R. Perez-Atayde, J.A. Fletcher, BRD4-NUT fusion oncogene: a novel mechanism in aggressive carcinoma, Cancer Res. 63 (2003) 304–307.
[8] C.A. French, J.L. Kutok, W.C. Faquin, J.A. Toretsky, C.R. Antonescu, C.A. Griffin, V. Nose, S.O. Vargas, M. Moschovi, F. Tzortzatou-Stathopoulou, I. Miyoshi, A.R. Perez-Atayde, J.C. Aster, J.A. Fletcher, Midline carcinoma of children and young adults with NUT rearrangement, J. Clin. Oncol. 22 (2004) 4135–4139.
[9] C.A. French, C.L. Ramirez, J. Kolmakova, T.T. Hickman, M.J. Cameron, M.E. Thyne, J.L. Kutok, J.A. Toretsky, A.K. Tadavarthy, U.R. Kees, J.A. Fletcher, J.C. Aster, BRD-NUT oncoproteins: a family of closely related nuclear proteins that block epithelial differentiation and maintain the growth of carcinoma cells, Oncogene 27 (2008) 2237–2242.
[10] C.A. French, NUT midline carcinoma, Cancer Genet. Cytogenet. 203 (2010) 16–20.
[11] H. Haack, L.A. Johnson, C.J. Fry, K. Crosby, R.D. Polakiewicz, E.B. Stelow, S.M. Hong, B.E. Schwartz, M.J. Cameron, M.A. Rubin, M.C. Chang, J.C. Aster, C.A. French, Diagnosis of NUT midline carcinoma using a NUT-specific monoclonal antibody, Am. J. Surg. Pathol. 33 (2009) 984–991.
[12] N. Haruki, K.S. Kawaguchi, S. Eichenberger, P.P. Massion, A. Gonzalez, A.F. Gazdar, J.D. Minna, D.P. Carbone, T.P. Dang, Cloned fusion product from a rare t(15;19)(q13.2;p13.1) inhibit S phase in vitro, J. Med. Genet. 42 (2005) 558–564.
[13] M.S. Hsieh, C.A. French, C.W. Liang, C.H. Hsiao, NUT midline carcinoma: case report and review of the literature, Int. J. Surg. Pathol. 19 (2011) 808–812.
[14] I. Kubonishi, N. Takehara, J. Iwata, H. Sonobe, Y. Ohtsuki, T. Abe, I. Miyoshi, Novel t(15;19)(q15;p13) chromosome abnormality in a thymic carcinoma, Cancer Res. 51 (1991) 3327–3328.
[15] H. Kurokawa, M. Zhang, S. Matsumoto, Y. Yamashita, T. Tanaka, K. Takamori, K. Igawa, M. Yoshida, H. Fukuyama, T. Takahashi, S. Sakoda, Reduced syndecan-1 expression is correlated with the histological grade of malignancy at the deep invasive front in oral squamous cell carcinoma, J. Oral Pathol. Med. 35 (2006) 301–306.
[16] T. Kuzume, I. Kubonishi, S. Takeuchi, T. Takeuchi, J. Iwata, H. Sonobe, Y. Ohtsuki, I. Miyoshi, Establishment and characterization of a thymic carcinoma cell line (Ty-82) carrying t(15;19)(q15;p13) chromosome abnormality, Int. J. Cancer 50 (1992) 259–264.
[17] A. Martinez, M.L. Spencer, U. Brethauer, P. Grez, F.J. Marchesani, I.G. Rojas, Reduction of syndecan-1 expression during lip carcinogenesis, J. Oral Pathol. Med. 38 (2009) 580–583.
[18] T. Soukka, J. Pohjola, P. Inki, R.P. Happonen, Reduction of syndecan-1 expression is associated with dysplastic oral epithelium, J. Oral Pathol. Med. 29 (2000) 308–313.
[19] E.B. Stelow, A.M. Bellizzi, K. Taneja, S.E. Mills, R.D. Legallo, J.L. Kutok, J.C. Aster, C.A. French, NUT rearrangement in undifferentiated carcinomas of the upper aerodigestive tract, Am. J. Surg. Pathol. 32 (2008) 828–834.
[20] E.B. Stelow, A review of NUT midline carcinoma, Head Neck Pathol. 5 (2011) 31–35.

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[21] H. Sugimura, Detection of chromosome changes in pathology archives: an application of microwave-assisted fluorescence in situ hybridization to human carcinogenesis studies, Carcinogenesis 29 (2008) 681–687.
[22] H. Sugimura, H. Mori, K. Nagura, S. Kiyose, H. Tao, M. Isozaki, H. Igarashi, K. Shinmura, A. Hasegawa, Y. Kitayama, F. Tanioka, Fluorescence in situ hybridization analysis with a tissue microarray: 'FISH and chips' analysis of pathology archives, Pathol. Int. 60 (2010) 543–550.
[23] M. Tanaka, K. Kato, K. Gomi, M. Yoshida, T. Niwa, N. Aida, H. Kigasawa, Y. Ohama, Y. Tanaka, NUT midline carcinoma: report of 2 cases suggestive of pulmonary origin, Am. J. Surg. Pathol. 36 (2012) 381–388.

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Our results are in line with the findings of Schmidt *et al.* (2009) who conclude that apoptosis is not required for pemphigus acantholysis. Like us, they found no evidence of positive cl caspase 3 and TUNEL when analyzing lesional PV patient skin. Moreover, in cultures of human keratinocytes treated with PV IgG, they also found acantholysis in absence of positive TUNEL staining as well as caspase 3 cleavage. Lee *et al.* (2009) also disagreed that apoptosis is an upstream event in pemphigus acantholysis. In PF IgG–treated mice and PV IgG–treated keratinocyte cultures, they found activation of cl PARP, cl caspase 3, and TUNEL, but only after acantholysis.

The belief that apoptosis is an upstream event in acantholysis is largely based on studies in model systems (Schmidt and Waschke, 2009). We question whether these model systems are suitable for answering such questions on pemphigus pathogenesis, as they differ from the *in vivo* situation. For instance, in cultured keratinocytes the desmosomal makeup in terms of molecular composition does not accurately reflect the *in vivo* situation (van der Wier et al., 2010). As for mouse models, the repertoire of expressed genes involved in apoptosis in humans and mice is different (Reed *et al.*, 2003). Furthermore, previous data on pemphigus skin are scarce and based on a few lesional biopsies only (Wang *et al.*, 2004; Pacheco-Tovar *et al.*, 2009, Deyhimi and Tavakoli, 2012).

In conclusion, this study does not support the hypothesis that apoptosis is involved in pemphigus acantholysis. However, although we studied morphological hallmarks of apoptosis, the involvement of certain apoptotic caspases or signaling pathways, which might be involved in dissociation of (inter)desmosomal adhesion complexes, cannot be excluded (Grando *et al.*, 2009).

CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

de Boer RA, van Veldhuisen DJ, van der Wijk J *et al.* (2000) Additional use of immunostaining for active caspase 3 and cleaved actin and PARP fragments to detect apoptosis in patients with chronic heart failure. *J Card Fail* 6:330–7

Deyhimi P, Tavakoli P (2012) Study of apoptosis in oral pemphigus vulgaris using immunohistochemical marker bax and TUNEL technique. *J Oral Pathol Med* 42:409–14

Grando SA, Bystryn JC, Chernyavsky AI *et al.* (2009) Apoptolysis: a novel mechanism of skin blistering in pemphigus vulgaris linking the apoptotic pathways to basal cell shrinkage and suprabasal acantholysis. *Exp Dermatol* 18:764–70

Hengartner MO (2000) The biochemistry of apoptosis. *Nature* 407:770–6

Lee HE, Berkowitz P, Jolly PS *et al.* (2009) Biphasic activation of p38MAPK suggests that apoptosis is a downstream event in pemphigus acantholysis. *J Biol Chem* 284:12524–32

Oktarina DA, van der Wier G, Diercks GF *et al.* (2011) IgG-induced clustering of desmogleins 1 and 3 in skin of patients with pemphigus fits with the desmoglein nonassembly depletion hypothesis. *Br J Dermatol* 165:552–62

Pacheco-Tovar D, Lopez-Luna A, Herrera-Esparza R *et al.* (2011) The caspase pathway as a possible therapeutic target in experimental pemphigus. *Autoimmune Dis* 2011:563091

Pacheco-Tovar MG, Avalos-Diaz E, Vega-Memije E *et al.* (2009) The final destiny of acantholytic cells in pemphigus is fas mediated. *J Eur Acad Dermatol Venereol* 23:697–701

Reed JC, Doctor K, Rojas A *et al.* (2003) Comparative analysis of apoptosis and inflammation genes of mice and humans. *Genome Res* 13:1376–88

Schmidt E, Gutberlet J, Siegmund D *et al.* (2009) Apoptosis is not required for acantholysis in pemphigus vulgaris. *Am J Physiol Cell Physiol* 296:C162–72

Schmidt E, Waschke J (2009) Apoptosis in pemphigus. *Autoimmun Rev* 8:533–7

Taatjes DJ, Sobel BE, Budd RC (2008) Morphological and cytochemical determination of cell death by apoptosis. *Histochem Cell Biol* 129:33–43

van der Wier G, Pas HH, Jonkman MF (2010) Experimental human cell and tissue models of pemphigus. *Dermatol Res Pract* 2010:143871

Wang X, Bregegere F, Frusic-Zlotkin M *et al.* (2004) Possible apoptotic mechanism in epidermal cell acantholysis induced by pemphigus vulgaris autoimmunoglobulins. *Apoptosis* 9:131–43

Associations between Keloid Severity and Single-Nucleotide Polymorphisms: Importance of rs8032158 as a Biomarker of Keloid Severity

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TO THE EDITOR

A keloid is a fibroproliferative disorder of the skin (Tredget, 1994) that is caused by pathological wound-healing processes (Gurtner *et al.*, 2008). The development and progression of keloids relate closely to genetic factors (Marneros *et al.*, 2004; Nakashima *et al.*, 2010), systemic factors such as hypertension (Arima *et al.*, 2012) and pregnancy (Park and Chang 2012), and the local microenvironment (e.g., skin stretching force/tension, (Ogawa *et al.*, 2012a)). A genome-wide association

Abbreviations: CI, confidence interval; GWAS, genome-wide association study; NEDD4, neuronal precursor cell-expressed developmentally downregulated 4; SNP, single-nucleotide polymorphism

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