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Commentary Open Access

Sexy Small Copy Numbers in Hereditary Gastric Carcinogenesis

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Commentary

Occurrence of gastric cancer in a family has been known for many years, and since the discovery of germline mutations of CDH1 in Maori families [1], the entity of hereditary diffuse gastric cancer (HDGC) (OMIM #137215) has been almost established. The guidelines of diagnosis and management have been regularly updated [2-5]. In many countries including Japan, where gastric cancer is endemic, the search and surveillance of the family has been continued. Reviewing the publications for the past two decades, the progress is not rapid, but considerably steady. New mutations are coming out from many countries every few months. The cases do not always fulfill the criteria of age and family histories; some occurred in the older patients [6], and the others occur in the patients without any family history [7-9]. The reports on the related diseases, lobular carcinoma of the breast [10], prostate cancer [11], colorectal cancer [12], and cleft lips [13,14] are also among the literatures.

I would like to draw the readers' attention to four reports recently published in the journals related to gastroenterology. They are on the germline alterations of CDH1 copy numbers in diffuse hereditary gastric cancer. With an introduction of Multiplex ligation-dependent probe amplification (MLPA) [15] method to CDH1 germline test, Oliveira et al. [16] identified exon-size CDH1 deletions in 6 probands. The sizes of the deletions encompassed 5'UTR-exon1 (1 family), exon1 to exon2 (3 families), exon14 to exon16 (1 family), and exon16 (1 family) of the CDH1. Next, we reported a case of exon3 deletion in Japanese family [17]. Japan has been notorious for being endemic with gastric cancer and a pursuit for HDGC was difficult because the application of the consensus guidelines to recommend genetic test pick up too many cases. The pessimism has been rampant on the prevalence of CDH1 germline changes in Japanese familial gastric cancer for the last 15 years especially in the community of clinicians [18]. The improved detection methods, easier sequencing (no more single-strand conformation polymorphism) covering whole exons and MLPA, re-motivated the attending clinicians such as endocsopists, surgeons, and attending pathologists to refer to genetic test labs when they fell any peculiarities in cases. The case referred to us 2 years after the first publication of a Japanese copy number deletion case was prominent. The multiple signet ring cell carcinoma occurred in a 30's without any family history of gastric cancer. Since the attending pathologists noticed that the features of the case resembled to the ones of HDGC [19], they asked us on testing for them. The sequencing of the whole exons of CDH1 and MLPA analysis was performed. The exon 11 of CDH1 was deleted in the germline of this proband and in his son. De novo occurrence of this deletion was confirmed by showing absence of it in his parents. Importantly his son succeeded it from his father [20] and surveillance program was considered. Just after this report, another very impressive case was reported from National Cancer Center Hospital, Tokyo appeared [21]. They applied array-comparable genomic hybridization (CGH) technology (Agilent Technologies, Santa Clara, CA) to the cases (the proband and his mother, a 56 years old post operative recurrence case) and discovered 275 kb deletion encompassing exon 7 to 16 of CDH1, a largest deletion in HDGC so far demonstrated (Table 1).

Authors	Ethnic origin	Cancer type	Copy number changes Ordered by the exons of CDH1
Oliveria et al. (16)	Southern European	HDGC	Deletion of 5'-UTR-exon 1
Oliveria et al. (16)	Northern European	HDGC	Deletion of exon 1-2
Oliveria et al. (16)	Canadian	HDGC	Deletion of exon 1-2
Oliveria et al. (16)	Eastern European	HDGC	Deletion of exon 1-2
Yamada H et al. (17)	Japanese	HDGC	Deletion exon 3
Benusiglio et al. (10)	French	LBC	Deletion exon 3
Yamada M et al. (21)	Japanese	HDGC	Deletion of exon 7-16
Sugimoto et al. (20)	Japanese	Early-onset DGC	Deletion of exon 11
Benusiglio et al. (10)	French	HDGC	Deletion of exon 11
Oliveria et al. (16)	Central European	HDGC	Deletion of exon 14-16
Oliveria et al. (16)	Central European	HDGC	Deletion of exon 16

Table 1: Summary of the cases with germline copy number change in CDH1 gene; HDGC: hereditary diffuse gastric cancer; DGC: diffuse gastric cancer; LBC: lobular breast carcinoma

The lesson we experienced is obvious. Our methods are still insufficient to recover all the deleterious genetic alterations from the subjects who daily visit the clinic; limitation of the access, lack of sharing the knowledge, and insufficient resource in genetics including history takers and counselors are hurdles in practical settings, even in Japan, where complete national health insurance coverage is famous. Insufficiency in our methods will be continued in front of us as to

identification of the genes besides CDH1, which are responsible for familial gastric cancer, more than 50% of them.

Usages of next generation sequencing (NGS) would be an obvious option for the institutes where the instruments and fund are available. In the recent American Association Cancer Research Annual Meeting in San Diego, two posters presented the first results of exome analyses. Identification of novel susceptibility genes in familial gastric cancer using next generation sequencing and identity-by-descent mapping; Genetic basis of hereditary gastric cancer: Beyond the CDH1 locus). Both and another Japanese group adopted whole exome analysis approaches, and we are now watching their validation and replication processes. Whole exome sequence (WES) will provide other gene mutations. Actually CTNNA1 mutation was identified using exome analysis [22] in a hereditary gastric cancer family. CTNNA1, a candidate gene associated with CDH1 in signal transduction, was not changed in another cohort of hereditary gastric cancer family [23]. This may cool-down our hyper-expectation on NGS (exactly WES) to identify new responsible genes. The genes found by WES may be sporadic or anecdotal ones.

Like the history of searches for CDH1 changes in Japan, the copy number changes in other gene loci would be possible culprit for familial occurrence of gastric cancer. Understanding the human genome variations [24] showed us there are numerous copy number variations in human genome. The copy numbers variations, from amylases to glutathione-S-transferases, are related to from our evolutionally fixed eating styles to inter-individual differences in xenobiotics adaptations, but the true significance of the most of them awaits further investigation. Inferring these genetic changes to the association of any disease require robust replication and control study. Actually some of the single nucleotide variations are found in our "control" DNAs (Table 2), thus the analyses of the "robust" controls are necessary. On the other hand some deletions (1-30 kb, small exonic CNVs) have been revealed to be responsible autism disorder [25]. Gastric cancer like autism spectrum disorders is relatively common, and occurs in modestly earlier stage of human life. It is obviously a very environmental disease according to the immigrant studies and the trends of the incidence observed in the past decades. Several platforms to survey the genome-wide alterations of copy numbers including NGS became available in genetic test labs for clinical use to detect large, moderately large, and exonic size deletions in the genome. When these platforms become more popular and economically feasible, the more numbers of endoscopists in practice are encouraged to refer any strange cases, such as early-onset, multiplicity, signet ring cell type, and of course, familial aggregation.

Nucleotide change	Amino acid change	Minor Allele Count	MAF	dbSNP ID	1000 Genomes*	
					Minor Allele Count	MAF
c.546A>C	p.Lys182Asn	1	0.003	rs201141645	1	0
c.2494G>A	p.Val832Met	2	0.005	rs35572355 ("pathogenic allele")	2	0.001
Duplication of exon 11	unknown	2	0.005	NA	NA	NA

Table 2: Single nucleotide variations of CDH1 gene found in 189 healthy aged Japanese controls (Yamada H et al. unpublished results) MAF: Minor allele frequency; NA: not available *http://www.1000genomes.org/announcements/may-2011-data-release-2011-05-12

At this moment, a greater question how this initiating genetic change brings about a full-blown gastric cancer remains to be investigated. Genetic and epigenetic changes at CDH1 locus are known, but the precise profile of the consequence in this particular disease, not like in common gastric cancer [26], awaits further investigation. The mouse model [27] and omics analysis including tumor microenvironment [28] may provide the clue.

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Teaching case

NSD3-NUT-expressing midline carcinoma of the lung: First characterization of primary cancer tissue

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ABSTRACT

Background: Nuclear protein in testis (NUT) midline carcinoma (NMC) is a rare, aggressive malignancy. Only two pediatric and three adult cases of pulmonary NMCs have been documented. In more than two-thirds of NMC cases, a gene fusion between *NUT* and *BRD4* or *BRD3* has been documented; other fusions are rare.

Case presentation: A 36-year-old woman was admitted because of a rapidly progressing tumor of the lung with metastases to the breast and bone. A biopsy from the lung tumor revealed an undifferentiated neoplasm exhibiting round to oval nuclei with vesicular chromatin, prominent nucleoli, and scant cytoplasm. Immunohistochemical staining demonstrated focal EMA, cytokeratin AE1/AE3, cytokeratin CAM 5.2, p63, CD138, and vimentin positivity. Finally, the nuclear staining pattern for NUT confirmed a histopathological diagnosis of NMC. A 5′- rapid amplification of the cDNA end (RACE) procedure successfully identified the partner of the *NUT* translocation as *NSD3*, a recently discovered partner. Fluorescence *in situ* hybridization confirmed the *NSD3-NUT* gene rearrangement, whereas a *BRD3/4-NUT* fusion gene was not detected.

Conclusion: We herein describe the first case of an NSD3-NUT-expressing NMC of the lung. The further accumulation of variant NMCs should provide clues to the establishment of new individualized therapy for NMCs.

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Introduction

Overall, malignant tumors of the lung that present with an undifferentiated morphology pose significant diagnostic challenges to attending surgical pathologists, especially in cases where a limited amount of biopsy material is available [20] and the immunohistochemical results are ambiguous or atypical [21]. Among these malignant neoplasms, nuclear protein in testis (NUT) midline carcinoma (NMC) is a recently recognized entity that is characterized by undifferentiated morphological features and immunoreactivity to NUT. This disease entity is notorious for its poor prognosis [1,16].

NMCs of the lung have been reported, but they are extremely rare; only 5 cases, including 2 pediatric [20] and 3 adult [7,21] cases, have been documented. Currently, the diagnosis of NMC depends on the identification of a genetic change (a rearrangement involving the *NUT* locus at 15q14) that generates a specific fusion transcript with a member of the bromodomain-containing protein (BRD) family, such as *BRD4* located on chromosome 19p13.1. In approximately two-thirds of NMCs [6,8,9], *BRD4-NUT* is specifically detected. Less commonly, NMC can harbor a different rearrangement involving *NUT* [9]; that is, a subset (approximately 25%) of these cases exhibit a *BRD3-NUT* rearrangement. In the remaining cases, the genes involved in *NUT* rearrangement are unknown [9]. For example, fluorescence *in situ* hybridization (FISH) has demonstrated a *BRD4-NUT* rearrangement in 2 pediatric cases of NMC of the lung [20]. In the remaining 3 adult cases of NMC arising in the lung, although *NUT* rearrangements were suspected because of break-aparts of the *NUT* locus in the cancer cells, the partner gene, supposed to be fused with *NUT*, was not identified

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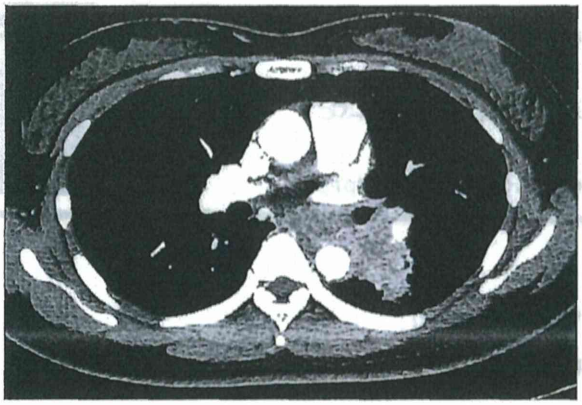

Fig. 1. Enhanced computed tomography scan performed at the time of hospitalization revealing a mass in the left lung.

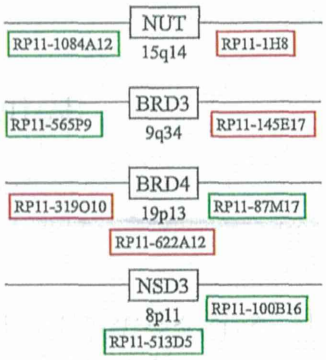

Fig. 2. Chromosomal positions of FISH probes flanking or including the genes of interest (*NUT*, *BRD3*, *BRD4*, and *NSD3*).

[7,21]. To date, no "variant NMC" of the lung harboring a fusion other than *BRD3/4-NUT* has been reported.

Recently, a variant NMC cell line (1221) was established, for the first time, from the discarded lung tumor tissue of a poorly differentiated squamous cell carcinoma of the mediastinum that had metastasized to the lung [10]. Using this cell line, a novel *NSD3* (*nuclear receptor binding SET domain 3*)-*NUT* fusion oncogene, which is both necessary and sufficient for the blockade of the differentiation and maintenance of proliferation in NMC cells, has been demonstrated [10]. Thus, NSD3 is a potential therapeutic target in NMC and a clinical example exhibiting this situation at a primary site has been sought.

In the present article, we describe the first case of an *NSD3-NUT* rearrangement identified in the primary tissue of an NMC of the lung; the NMC was diagnosed using immunohistochemistry with a highly sensitive and specific anti-NUT monoclonal antibody [11]. In addition, a *NSD3-NUT* fusion gene was successfully identified using 5′-rapid amplification of the cDNA end (RACE) and was validated using FISH.

Clinical summary

A 36-year-old woman sought medical advice because of a cough accompanied by wheezing with a 2-month duration. An enhanced computed tomography scan performed at the time of hospitalization revealed a tumor surrounding the proximal part of the left lower lobe bronchus with obstruction of the left bronchus B6 and extending to the middle mediastinum adjacent to the left hilum (Fig. 1), strongly indicating that the tumor was derived from the left lung rather than from the mediastinum. Metastatic lesions were revealed in the liver, breast, bones and lymph nodes. A biopsy was performed from the lung tumor and the lymph node.

The patient has been treated with chemoradiation therapy, including a regimen of cisplatin and docetaxel and radiotherapy for metastatic lesions, for 9 months since her diagnosis. The outcome was once partial response, however, eventually the status became disease progression at the point of 9 months after the diagnosis. The patient did not undergo targeted therapies.

Materials and methods

Histopathologic and immunohistochemical examination

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 DAKO autostainer universal staining system (DAKO, Glostrup, Denmark) with antibodies for the following antigens: CD5 (clone 4C7, 1:150 dilution, DAKO), CD30 (clone Ber-H2, 1:100, DAKO), CD45 (clone 2B11 + PD7/26, 1:150, DAKO), CD56 (clone CD564, 1:100, Leica Biosystems, Newcastle, United Kingdom), CD99 (clone 12E7, 1:100, DAKO), CD138/Syndecan-1 (clone MI15, 1:100, DAKO), CEA (poly, 1:600, DAKO), chromogranin A (poly, 1:100, DAKO), c-kit (poly, 1:100, DAKO), cytokeratin AE1/AE3 (clone AE1 + AE3, 1:100, DAKO), cytokeratin CAM5.2 (clone CAM5.2, prediluted by manufacturer, Becton, Dickinson and Company, CA, USA), desmin (clone D33, 1:100, DAKO), epithelial membrane antigen (EMA, clone E29, 1:150, DAKO), myogenin (clone F5D, 1:50, DAKO), NUT (C52B1, 1:25, Cell Signaling Technologies Inc., Danvers, MA, USA), p63 (clone 4A4, 1:100, Santa Cruz Biotechnology, Dallas, TX, USA), PLAP (clone 8A9, 1:100, Leica Biosystems), S-100 (poly, 1:1000, DAKO), synaptophysin (poly, 1:150, DAKO), TTF-1 (clone 8G7G3/1, 1:100, DAKO) and vimentin (clone V9, 1:200, DAKO).

FISH

The FISH procedure was performed as previously reported [17–19]. FISH probes were prepared from the Bacterial Artificial Chromosome (BAC) library. BAC probes flanking *NUT* (RP11-1084A12 and RP11-1H8), flanking *BRD3* (RP11-565P9 and RP11-145E17), flanking *BRD4* (RP11-319O10 and RP11-87M17), flanking *NSD3* (RP11-100B16), containing *BRD4* (RP11-622A12), and containing *NSD3* (RP11-513D5) were purchased from Advanced GenoTechs Co. (Tsukuba, Japan). All the probes were confirmed to be correct by hybridizing them to a metaphase human chromosome spread (data not shown). The RP11-1084A12, RP11-565P9, RP11-87M17, RP11-513D5 and RP11-100B16 probes were nick-translated using Green dUTP (2N32-50; Enzo Life Science, Farmingdale, NY, USA), and the RP11-1H8, RP11-145E17, RP11-319O10 and RP11-622A12 probes were nick-translated using Orange dUTP (2N33-50; Enzo Life Science). The colors and BAC probe RP numbers that were used are shown in Fig. 2. To detect the break-apart of *NUT*, *BRD3*, and *BRD4*, the RP11-1084A12, RP11-1H8; RP11-565P9, RP11-145E17; and RP11-319O10, RP11-87M17 probes were used, respectively. To detect the *BRD4-NUT* fusion, the combination of RP11-87M17 and RP11-1H8 was used. In addition, another combination of RP11-622A12 (containing BRD4) and RP11-1H8, the latter of which was nick-translated using Green dUTP (2N32-50) for this particular purpose, was used to detect the *BRD4-NUT* fusion for validation purposes. To detect the fusion of *BRD3-NUT*, the combination of RP11-1084A12 and RP11-145E17 was used. To detect *NSD3-NUT*, a mixture of RP11-513D5 (containing NSD3), RP11-100B16 (flank NSD3), and RP11-1H8 (flank NUT) was used.

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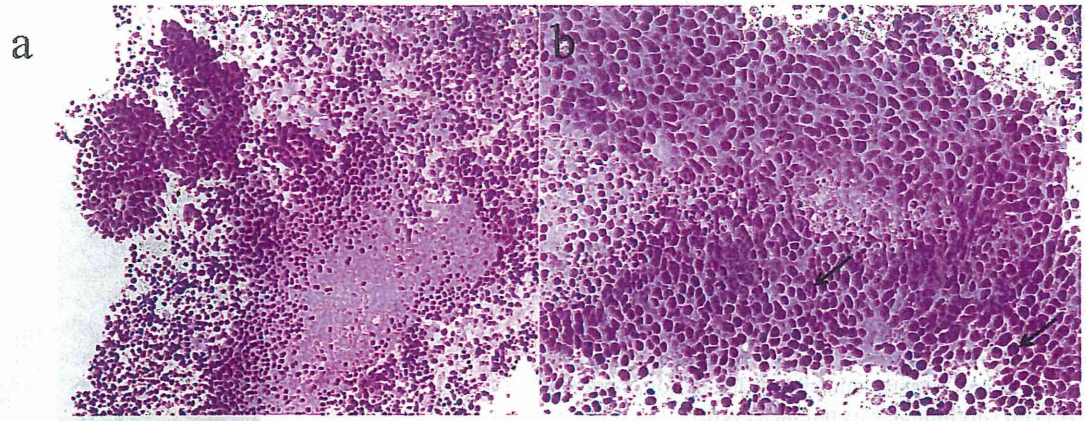

Fig. 3. Representative histologic images. (a) Sheets of undifferentiated malignant cells with focal necrosis are visible. (b) The tumor cells had round to oval nuclei with vesicular chromatin, prominent nucleoli, and scant cytoplasm. Frequent mitotic figures are visible (arrows).

The nuclei were stained with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). The slides were reviewed manually, with the examination of at least 50 tumor nuclei in each case. A cutoff of >30% nuclei showing a split or fused signal was considered a positive result indicating gene rearrangement.

5′-RACE

Total RNA was isolated from the patient's frozen tissue biopsy using the Isogen RNA extraction kit (Wako Pure Chemicals, Osaka, Japan) according to the manufacturer's instructions. One microgram of total RNA was used to amplify the 5′ end of the human *NUT* fusion mRNA using the Gene Racer kit (Invitrogen, Carlsbad, CA, USA). The *NUT* fusion mRNA was ligated with the Gene Racer™ RNA oligo and reverse-transcribed using SuperScript III reverse transcriptase (Life Science Technologies, Carlsbad, CA, USA) and the *NUT* exon 2 gene specific primer 5′-GCTTCTGTGTCAGGACTCTGGGATA-3′. The amplification of the 5′ ends of the *NUT* fusion cDNA was performed using Phusion polymerase (New England Biolabs, Beverly, MA, USA) with the Gene Racer 5′ Primer and the *NUT* exon 2 specific outer primer 5′-AACGCTGCCACTGACGGAAGTTCTC-3′. A nested PCR was also performed using the Gene Racer 5′

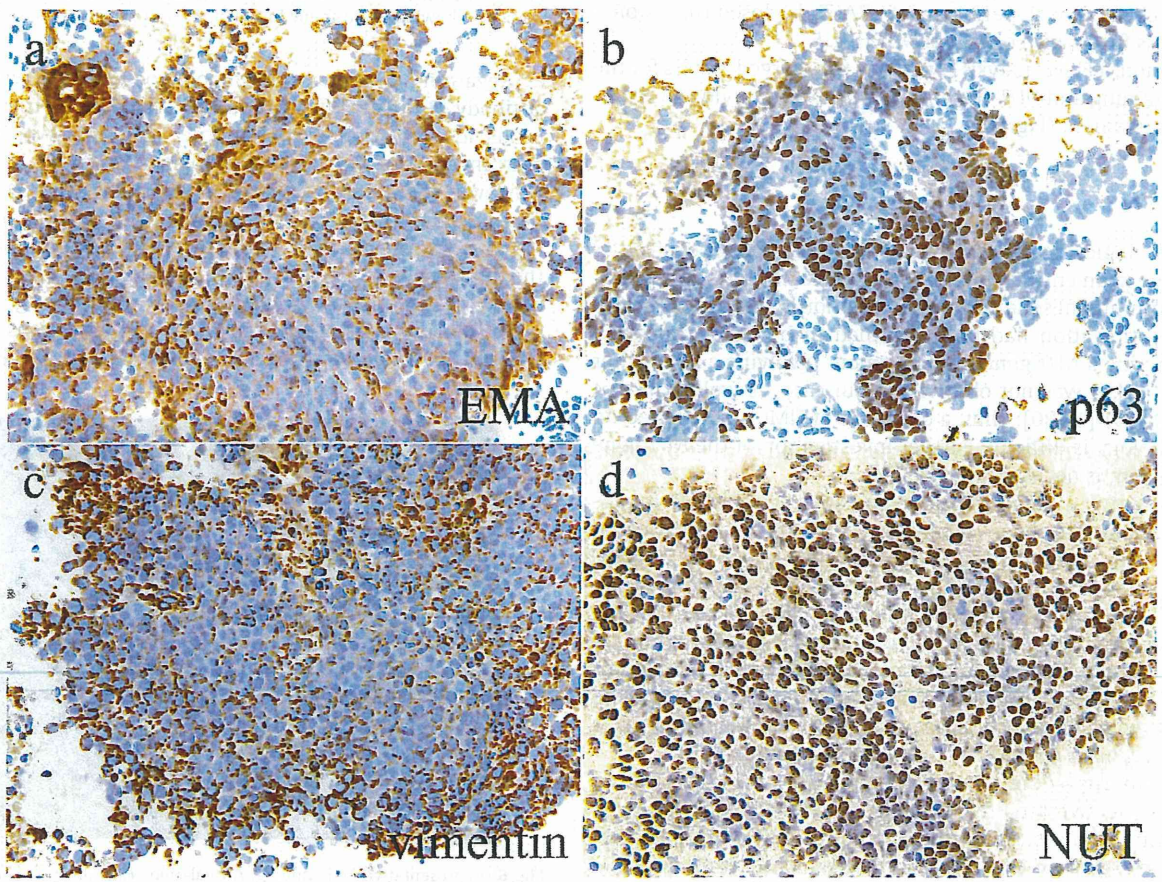

Fig. 4. Immunohistochemical findings of the NUT midline carcinoma. (a–c) The tumor cells are focally positive for EMA (a), p63 (b), and vimentin (c). (d) Immunohistochemistry shows a nuclear staining pattern for NUT.

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Nested Primer and the *NUT* exon 2 specific inner primer 5′-GAAGGCTTGGATAGAGTGGCCACAG-3′. The amplified PCR product was gel purified and directly sequenced using ABI PRISM Big Dye Terminators (Perkin-Elmer, Inc., Wellesley, MA, USA) and the ABI 3130 sequencer (Life Science Technologies).

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 (38/10 hpf) were observed (Fig. 3b, arrow). Squamous differentiation was not apparent. Rosette and ribbon formation, strongly suggesting small cell carcinoma, was not observed.

Immunohistochemical staining demonstrated focal positivity for EMA (Fig. 4a), p63 (Fig. 4b), cytokeratin AE1/AE3, cytokeratin CAM 5.2, CD138 and vimentin (Fig. 4c). Negative staining results were obtained for CD5, CD30, CD45, CD56, CD99, CEA, chromogranin A, c-kit, desmin, myogenin, PLAP, S-100, synaptophysin and TTF-1. In addition, a nuclear staining pattern for NUT was evident (Fig. 4d). These results confirmed a diagnosis of NMC.

Next, FISH was performed to confirm the rearrangement of *BRD3*, *BRD4*, and *NUT*. FISH analyses revealed the break-apart of *NUT*, but not the *BRD3* and *BRD4* genes. In addition, the merging of images showing *NUT*-flanking and *BRD3*-flanking or *BRD4*-containing BAC probes did not generate merged (in this case, yellow) signals. These findings suggested that this case should be temporarily assigned as a variant NMC, i.e., a case with an undefined rearrangement involving *NUT*.

To identify the gene involved in the *NUT*-variant rearrangement, a 5′-RACE analysis was performed. 5′-RACE disclosed that exon 7 of *NSD3* was fused with exon 2 of *NUT* (Fig. 5).

In addition, a FISH analysis confirmed the merged results for the *NSD3*-containing and *NUT*-flanking BAC probes, implying an *NSD3-NUT* rearrangement (Fig. 6).

Discussion

The histological features of tumors that have been reported as NMCs range from entirely undifferentiated carcinomas to carcinomas with prominent squamous differentiation [2–4,8,15]. Although abrupt keratinization had been assumed to be characteristic of NMC harboring a *NUT* gene rearrangement involving a gene other than *BRD4* [7], it was not observed in our case, as well as in that with *BRD4-NUT* [19,20]; thus, a diagnosis of NMC solely based on the morphology is difficult. Further investigation of the *NUT* gene rearrangement is necessary [20,21,23].

Very recently, a highly sensitive and specific anti-NUT monoclonal antibody has been established [11] and its commercial availability has made it possible to assess the prognosis of NMC amongst a larger number of patients [1,2]. A previous study using the largest cohort of NMC patients to date showed that the median overall survival period of patients was 6.7 months and no chemotherapeutic regimen improved the outcome [1]. Our patient underwent chemoradiotherapy, which failed to lead to complete remission and resulted in disease progression. Careful follow-up is needed to evaluate the effectiveness of this treatment.

Data from a growing number of identified cases can be used to develop effective targeted therapies. One candidate therapy is a bromodomain and extra terminal inhibitor (BETi) [5]. However, although one reported NMC case was subsequently enrolled at the first dose level of a phase 1 clinical trial of BETi, it failed to complete remission [13].

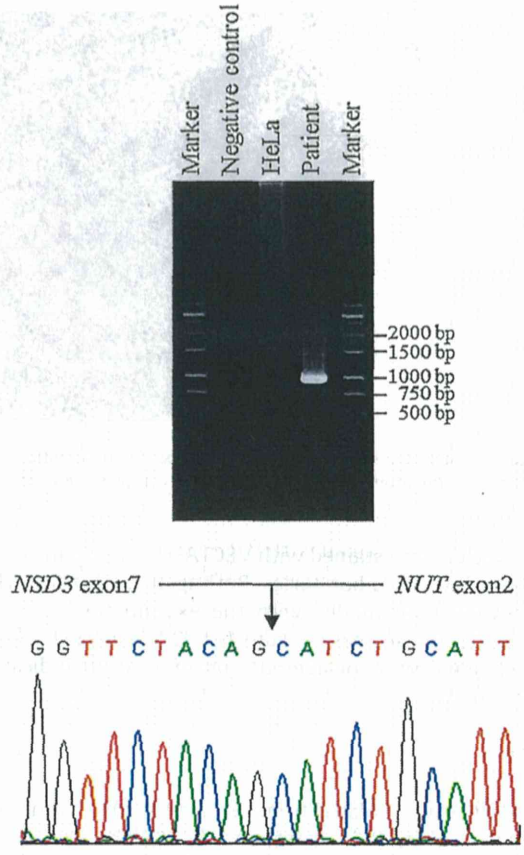

Fig. 5. Detection of *NSD3-NUT*. (a) PCR amplification with Gene Racer 5′ Nested Primer and the *NUT* exon 2 specific inner primer. (b) *NSD3-NUT* fusion junction sequence showing the in-frame fusion of *NSD3* exon7 with *NUT* exon2.

Regarding diagnosis, immunohistochemistry with anti-NUT antibody [11] can differentiate NMC from other tumors and rigorous identification of the partner gene involved in the *NUT* rearrangement is not essential for the diagnosis of NMC itself. However, considering the rapid development of drugs, the identification of new targets may provide new therapeutic opportunities for patients who otherwise have no other treatment options. Since the discovery of a somatic rearrangement involving the *NUT* gene in NMC [6], no study has successfully identified new genes involved in *NUT*-variant rearrangements in clinical specimens. In our case, 5′-RACE successfully identified a *NSD3-NUT* fusion gene, same as

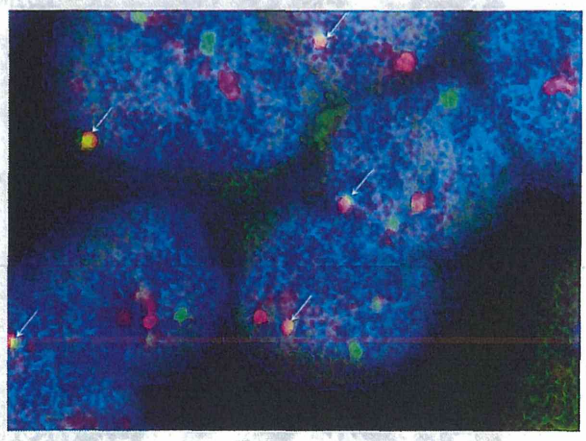

Fig. 6. Representative FISH finding. A dual-color FISH analysis shows the fusion gene as a single yellow (overlapping) signal (arrow), including a green (*NSD3*) and an orange (*NUT*) signal.

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that most recently demonstrated in a patient-derived variant NMC cell line, 1221 [10]. *NSD3* is reportedly amplified in breast cancer [12] and mutated in pancreas [22] and lung [22] cancers.

Histone modifying enzymes, including the NSD family, are often deregulated in cancer, and aberrant histone modification profiles are intimately linked to carcinogenesis [10,12]. In the literature, although one NMC case was treated with histone deacetylase inhibitors (HDACi), it failed to continue the therapy due to marked side effects [14]. On the other hand, inhibitors of histone methyltransferases (HMTase), including the NSD family, are also under development [12]. Such therapies hold promise for NMC. In addition, it is possible that combination therapy with HDACi, HMTase inhibitors, and BETi may be more effective than monotherapy for this aggressive disease.

In this article, we have described the first case of a variant NMC of the lung, providing information that is pertinent to the expansion of knowledge regarding this rare tumor. Furthermore, the identification of the *NSD3-NUT* fusion gene using 5′-RACE and diagnostic FISH in this tumor has provided new insight into the influence of this fusion on clinicopathological features, including the locations of tumors, the ethnicity of patients, the degree of squamous differentiation, and the responsiveness to new therapy. Further identifications of variant rearrangements involving *NUT*, such as the *NSD3-NUT* fusion reported in the present case, should pave the way to the establishment of new individualized therapies against this rare and ominous disease.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Full Paper

Association Between *KCNJ6* (*GIRK2*) Gene Polymorphism rs2835859 and Post-operative Analgesia, Pain Sensitivity, and Nicotine Dependence

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Abstract. G-protein–activated inwardly rectifying potassium (GIRK) channels are expressed in many tissues and activated by several $G_{i/o}$ protein–coupled receptors, such as opioid and dopamine receptors, and thus are known to be involved in the modulation of opioid-induced analgesia, pain, and reward. We focused on a GIRK-channel subunit that plays a pivotal role in the brain, GIRK2, and investigated the contribution of genetic variations of the *GIRK2* (*KCNJ6*) gene to individual differences in the sensitivity to opioid analgesia. In our initial linkage disequilibrium analysis, a total of 27 single-nucleotide polymorphisms (SNPs) were selected within and around the regions of the *KCNJ6* gene. Among them, the rs2835859 SNP, for which associations with analgesia and pain have not been previously reported, was selected in the exploratory study as a potent candidate SNP associated with opioid analgesic sensitivity. The results were corroborated in further confirmatory study. Interestingly, this SNP was also found to be associated with sensitivity to both cold and mechanical pain, susceptibility to nicotine dependence, and successful smoking cessation. The results indicate that this SNP could serve as a marker that predicts sensitivity to analgesic and pain and susceptibility to nicotine dependence.
[Supplementary materials: available only at http://dx.doi.org/10.1254/jphs.14189FP]

Keywords: G-protein–activated inwardly rectifying potassium (GIRK) channel, single-nucleotide polymorphism, opioid analgesia, pain, susceptibility to nicotine dependence

Introduction

G-protein–activated inwardly rectifying potassium (GIRK) channels are members of the inwardly rectifying potassium channel family, and four kinds of subunits (GIRK1-GIRK4) have been identified in mammals (1). GIRK channels are expressed in many tissues, including the heart (2), spinal cord (3, 4), and various regions in the brain with different subunit compositions (5 – 7). GIRK channel activation is triggered by the activation of several $G_{i/o}$ protein–coupled receptors, such as opioid (8), M_2-muscarinic (9), D_2- and D_4-dopaminergic (10), α_2-adrenergic (11), serotonin 1A (5-HT_{1A}) (12), metabotropic glutamate (13), somatostatin (14), CB_1-cannabinoid (15, 16), nociceptin/orphanin FQ (17), and A_1-adenosine (18) receptors. Neuronal GIRK channels are predominantly heteromultimeric, composed of GIRK1 and GIRK2 subunits in most brain regions (19, 20), or homomultimeric, composed of GIRK2 subunits in the substantia nigra (21). Several studies that used knockout mice showed that opioid-induced GIRK channel activation co-expressed with opioid receptors inhibited

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nociceptive transmission and thus opioid-induced analgesia (2, 3, 22 – 24). Furthermore, GIRK1-, GIRK2-, and GIRK3-knockout mice exhibited hyperalgesia in the hot-plate and tail-flick tests of thermal nociception (4, 23), suggesting the involvement of GIRK channels in the sensitivity to hot stimulus-induced pain. GIRK channels have also been reported to be involved in the rewarding effects of ethanol and cocaine in studies of GIRK2- and GIRK3-knockout mice (25, 26).

Among many related functions or phenotypes, the effects of GIRK channels on analgesia and pain perception mentioned above are mediated by upstream opioid signaling, which is known to play important roles in both antinociception and reward (27, 28). To date, only a few studies have examined the relationship between genetic variations in GIRK channels and phenotypic differences related to opioid action in humans (29 – 32). One of these studies was conducted by our group (31), in which we sought to reveal the relationship between single-nucleotide polymorphisms (SNPs) in the *KCNJ6* gene that encodes human GIRK2, especially within the exonic and 5′-flanking regions, and individual differences in opioid analgesic sensitivity. Another recent study found an association between *KCNJ6* SNPs and pain-related phenotypes, reconfirming that the *KCNJ6* gene is a promising target for investigating the genetic factors that contribute to pain and analgesia (29).

The present study sought to comprehensively reveal the relationship between SNPs in the *KCNJ6* gene region, including the intronic region, and individual differences in the sensitivity to analgesia, experimental pain, and smoking behavior.

Materials and Methods

Ethics statement

The study protocol was approved by the Institutional Review Boards at Tokyo Dental College, Chiba, Japan (Tokyo), Hamamatsu University School of Medicine (Hamamatsu), and the Tokyo Institute of Psychiatry (currently Tokyo Metropolitan Institute of Medical Science; Tokyo). All of the subjects provided informed, written consent for the genetics studies.

Subjects

Enrolled in the initial analysis to explore the association between *GIRK2* gene polymorphisms and the sensitivity to opioid-induced analgesia were 355 healthy patients who were scheduled to undergo cosmetic orthognathic surgery (mandibular sagittal split ramus osteotomy) for mandibular prognathism at Tokyo Dental College Suidoubashi Hospital, as described in the Supplementary Materials and Methods (available in the online version only) and a previous report (33). Peripheral blood samples were collected from these subjects for the gene analysis. The detailed demographic and clinical data of the subjects are provided in Supplementary Table 1 (available in the online version only).

The subjects used in the association study to examine the contribution of *GIRK2* gene polymorphisms to the sensitivity to pain were a total of 500 healthy volunteers who lived in the Kanto area of Japan, as described in the Supplementary Materials and Methods (age 20 – 72 years, 253 males, 242 females, and five gender-unknown subjects). Oral mucosa samples were collected from the subjects for the gene analysis. All of the subjects underwent the cold pressor–induced pain test (CPT) and mechanically induced pain test (MPT). Additionally, the Temperament and Character Inventory (TCI) (34 – 36), a self-report measure of temperament and character dimensions, was used to profile the personalities of these subjects. The detailed demographic and clinical characteristics of the subjects are provided in Supplementary Table 2 (available in the online version only).

Participants in the subsequent study to examine the association between *GIRK2* gene polymorphisms and the susceptibility to nicotine dependence included a total of 1,000 patients who visited Iwata City Hospital in Japan. The inclusion criteria for this study were being ambulatory, able to communicate orally, and 60 years of age or older. Numerous participants in this study had various smoking habits and completed a questionnaire that consisted of various questions about lifestyle, including alcohol consumption, smoking, diet, and cancer history (37). Peripheral blood samples were collected from these subjects for the gene analysis. The detailed demographic and clinical characteristics of the subjects are provided in Supplementary Table 3 (available in the online version only).

Data collection

For the subjects who underwent cosmetic orthognathic surgery, the surgical protocol and subsequent postoperative pain management were fundamentally the same as those of the previous study (33, 38) and detailed in the Supplementary Materials and Methods. Postoperative patient-controlled analgesia (PCA) fentanyl use during the first 24-h postoperative period was recorded. The dose of fentanyl administered postoperatively was normalized to body weight.

For the healthy volunteer subjects, the results from the two pain tests were recorded. The CPT was performed basically as previously described (39, 40), although a slight modification was made. Basal endpoint sensitivity to pain was evaluated as detailed in the Supplementary Materials and Methods (Supplementary Table 2). In the