Biochemical and Biophysical Research Communications 452 (2014) 980–985

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Frequent mutations in NOTCH1 ligand-binding regions in Japanese oral squamous cell carcinoma

Ken-ichi Aoyama [a,b], Yoshihide Ota [a], Kagemasa Kajiwara [b], Noriaki Hirayama [b], Minoru Kimura [b,*]

[a] Department of Oral and Maxillofacial Surgery, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan
[b] Department of Molecular Life Science, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan

ARTICLE INFO

Article history:
Received 1 September 2014
Available online 16 September 2014

Keywords:
Japanese oral squamous cell carcinoma
NOTCH1
Point mutation
Ligand binding region
Protein structure simulation

ABSTRACT

Recent studies showed that head and neck squamous cell carcinoma (HNSCC) including oral squamous cell carcinoma (OSCC) of Caucasian, Chinese and Indian patients frequently have NOTCH1 mutations. We found eight of 84 OSCC in Japanese patients have point mutations (9.5%) correspond to the ligand binding region of NOTCH1 protein. Two set of them are the same mutations and all mutations are non-synonymous G > A transitions. In addition, median disease-free survival is significantly longer in patients with NOTCH1-mutated tumors as compared to those without the mutation ($P < 0.05$). The protein structure simulation based on X-ray crystallography indicated that new p.A465T mutation leads to a conformational change of NOTCH1 ligand binding domain as well as the p.G481S mutant NOTCH1 with a loss of flexibility around this residue. These results suggest that *NOTCH1* mutation occurs frequently in Japanese OSCC in the vicinity of the ligand binding region and, these mutations cause downregulation of the NOTCH1 function.

1. Introduction

Oral squamous cell carcinoma (OSCC) is one of the sixth most common cancer in the world [1]. The incidence and mortality from the disease vary geographically. In India, Bangladesh, Pakistan, and Sri Lanka, OSCC is the most common cancer, accounting for about one-third of all cancers [2]. Although a number of prognostic factors have been implicated to date [3], there is no biological marker for OSCC. Several exome sequencing studies have examined Caucasian head and neck squamous cell carcinoma (HNSCC) occurring at a heterogeneous set of anatomical sites, including OSCC [3–6], and found frequent *NOTCH1* mutations, in addition to some mutations known in solid cancers such as *TP53*, *CDKN2A*, *PIK3CA* and *HRAS* [4–7]. High frequencies of *NOTCH1* mutations were also observed in Chinese OSCC [8].

NOTCH protein is a single-pass transmembrane receptor. Mammalian NOTCH family has four subtypes (termed NOTCH1–4), and affects cell–cell interaction, proliferation, differentiation and apoptosis of diverse types of cell in a variety of organisms [9]. NOTCH receptor consists of the NOTCH extracellular fragment domain (NECD), transmembrane and the NOTCH intracellular fragment. NECD consists of epidermal growth factor (EGF)-like repeats 1–36. EGF-like repeats 10–12 are important and sufficient for binding of Jagged and DLL family ligands [10–13]. After ligand binding, NOTCH receptor is cleaved by gamma secretase complex and releases NICD into the nucleus. NICD interacts with DNA-binding protein RBPJ and the resulting complex activates Hes or Hey family of transcription genes, thereby promoting downstream programs [14].

NOTCH proteins regulate various oncogenes and tumor suppressor genes, such as *c-myc*, *PI3K*, *EGFR*, *PTEN*, and *TP53*. Therefore, NOTCH disorder may play a dual role in tumorigenesis. For example, both overexpression and downregulation of NOTCH and ligands have been implicated in several human cancers [15–18]. The first indication that NOTCH promotes tumorigenesis came from the identification of a chromosomal translocation in a subset of human T-cell acute lymphoblastic leukemia (TALL) [17]. Subsequently, activating mutations in NOTCH1 NICD were discovered in more than 50% of human TALL [15]. For OSCC, there are some *in vitro* studies [19,20], but the function of NOTCH1 for tumorigenesis is unsettled.

Abbreviations: OSCC, oral squamous cell carcinoma; HNSCC, head and neck squamous cell carcinoma; NECD, NOTCH extracellular domain; NICD, NOTCH intracellular domain; TALL, T-cell acute lymphoblastic leukemia; EGF, epidermal growth factor; T stage, tumor stage; N stage, lymph node metastasis stage; OS, overall survival; DFS, disease free survival.

* Corresponding author. Fax: +84 463 96 2892.
E-mail address: kimura@is.icc.u-tokai.ac.jp (M. Kimura).

http://dx.doi.org/10.1016/j.bbrc.2014.09.021
0006-291X/

In this study, we examined the sequence of *NOTCH1* ligand binding region in Japanese OSCC and investigated the clinical significance of mutations as a prognostic marker.

2. Materials and methods

2.1. OSCC cell lines

The study included 6 OSCC cell lines. KON, HSC-3, HSC-4, OSC-20, Ca9-22 were kind gifts from Dr. Takeshi Onda (Tokyo Dental College, Tokyo, Japan) and SAS was purchased from Riken Cell Bank (Tsukuba, Ibaraki, Japan).

2.2. Patients and clinical samples

Tumors and oral mucosa tissues were collected between April 2010 and November 2012 at the time of surgical resection from 84 treatment-naive OSCC patients. The patients had a median age of 70.5 years at diagnosis and underwent a comprehensive staging, radical resection, post-operative radiotherapy with or without chemotherapy and follow-up from 1.9 years to 4.4 years in the Department of Oral and Maxillofacial Surgery, Tokai University Hospital. Pathologists distinguished in a blind manner whether the collected tissues were tumor or normal tissues.

Overall survival (OS) was defined as the time from the date of diagnosis to the date of death or the last follow-up. Disease-free survival (DFS) was defined as the time from surgery to disease relapse.

This study was approved by the ethics committee of the Tokai University School of Medicine (No.12 I-48). Informed consent was obtained from all patients according to the Declaration of Helsinki.

2.3. DNA extraction

Tissue samples were digested with proteinase K in a buffer consisting of 150 mM NaCl, 10 mM Tris–HCl (pH 8.0), 10 mM EDTA and 0.1% SDS. Genomic DNA was obtained by phenol chloroform extraction followed by ethanol precipitation and stored in 10 mM Tris–HCl (pH 8.0) containing 1 mM EDTA at −70 °C.

2.4. PCR and DNA sequencing

The entire coding region of *NOTCH1* was sequenced in OSCC cell lines. In clinical samples, we examined *NOTCH1* exon 6–9 corresponding to EGF-like repeats 10–13. Primer pairs for PCR amplification and sequencing were designed using Primer3 (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). The primer sequences are shown in Supplementary Data. PCR was performed for 35 cycles in a total volume of 20 μL containing 7.5 ng of extracted DNA as a starting template. The PCR mixture contained 1.0 units KOD FX neo (TOYOBO, Osaka, Japan), 8.0 mM $MgCl_2$, 2.0 mM dNTP, and 0.3 μM primers (forward and reverse). PCR products were purified and sequenced using Bigdye XTerminator® (Lifetechnology, Carlsbad, USA) on the Applied Biosystems 3500 Genetic Analyzer. Sequences were analyzed by Sequencing Analysis Software Version 5.4 (Lifetechnology). Variants were annotated using gene structure from the NCBI RefSeq (NG_007458.1) transcript set.

2.5. Simulation of the 3D structure for NOTCH1 mutants

We simulated the structure of mutated NOTCH1 EGF-like repeats 11–13 using molecular mechanics calculations based on the high-resolution X-ray crystal structure of the ligand binding domain of NOTCH1 (PDB: 2VJ3) [21]. A software system, Molecular Operating Environment (Chemical Computing Group, Montreal, Quebec, Canada) was used.

2.6. Statistical analysis

Continuous and categorical variables were compared using Fisher's exact test and the chi-square test. Survival curves were estimated using the Kaplan–Meier method. All statistical analyses were performed using the SPSS program (version 22), and $P < 0.05$ was considered significant.

3. Results

3.1. Patient characteristics

Clinical characteristics of 84 OSCC patients are shown in Table 1. Overall, median DFS was significantly shorter for patients treated with adjuvant therapy comprising radiotherapy with or without chemotherapy than for those treated with surgery alone (30.2 vs.

Table 1
Patient clinicopathological characteristics.

Characteristics	Patients		
	Total	NOTCH1 mutation	
		Yes	No
	n = 84	*n* = 8	*n* = 76
Age (years)			
	69 ± 11.5	74.4 ± 7.7	68.7 ± 11.8
Sex			
Men	51 (60.7)	7 (87.5)	44 (57.9)
Women	33 (39.3)	1 (12.5)	33 (42.1)
Localization			
Tongue	30 (35.7)	2 (25.0)	28 (36.8)
Mandibular gingiva	28 (33.3)	1 (12.5)	27 (35.5)
Maxillary gingiva	10 (11.9)	2 (25.0)	8 (10.5)
Buccal	8 (9.5)	1 (12.5)	7 (9.2)
Floor	4 (4.8)	1 (12.5)	3 (3.9)
Hard palate	3 (3.6)	1 (12.5)	2 (2.6)
Lip	1 (1.2)	0 (0)	1 (1.3)
Histological differentiation			
Well	49 (58.3)	7 (87.5)	42 (55.3)
Moderate	30 (35.7)	1 (12.5)	29 (38.2)
Poor	5 (6.0)	0 (0)	5 (6.6)
T stage			
T1	25 (29.8)	5 (62.5)	20 (26.3)
T2	24 (28.6)	0 (0)	24 (31.6)
T3	6 (7.1)	0 (0)	6 (7.9)
T4	29 (34.5)	3 (37.5)	26 (34.2)
N stage			
N0	46 (54.8)	5 (62.5)	41 (53.9)
N1	13 (15.5)	0 (0)	13 (17.1)
N2a	0 (0)	0 (0)	0 (0)
N2b	19 (22.6)	3 (37.5)	16 (21.1)
N2c	6 (7.1)	0 (0)	6 (7.9)
N3	0 (0)	0 (0)	0 (0)
Extracapsular spread lymph node metastasis			
Yes	75 (89.3)	7 (87.5)	68 (89.5)
No	9 (10.7)	1 (12.5)	8 (10.5)
Treatment			
Surgery only	51 (60.7)	4 (50.0)	47 (61.8)
Adjuvant RT	11 (13.1)	3 (37.5)	8 (10.5)
Adjuvant CCRT	19 (22.6)	1 (12.5)	18 (23.7)
Adjuvant CTX	3 (3.6)	0 (0)	3 (3.9)

Values are means ± SD or number (%) of patients.
Abbreviations: CCRT, concurrent chemoradiation therapy; CTX, chemotherapy; RT, radiotherapy.

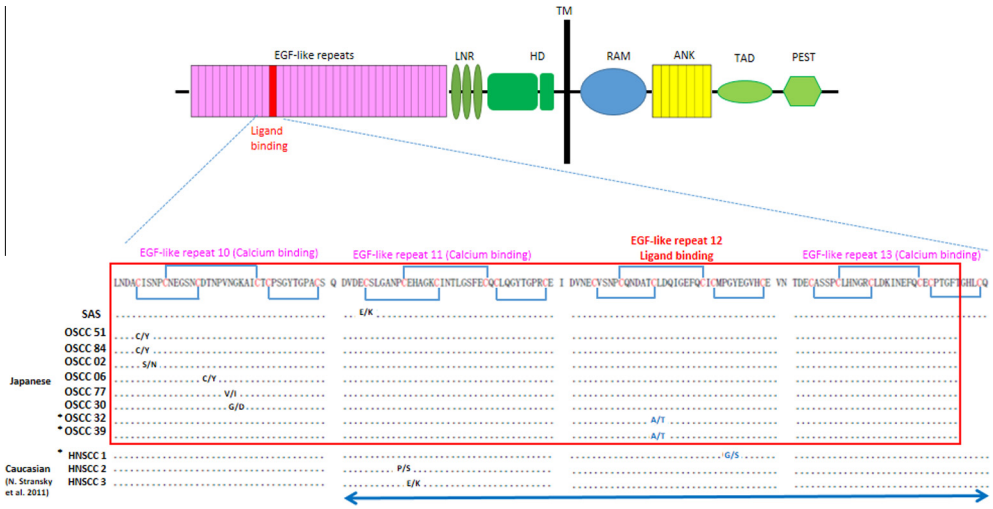

Fig. 1. The map of point mutations found in Japanese OSCC. Red square corresponds to the sequenced area of NOTCH1 in Japanese OSCC. The high-resolution X-ray crystal structure of the ligand binding region was registered in protein data base by Cordle et al. (PDB: 2VJ3) (shown by blue arrow). Disulfide bonds are shown by light blue line. * : 3D simulation for these mutants are shown in Fig. 3. Abbreviations: EGF, epidermal growth factor; LNR, Lin/NOTCH repeats; HD, heterodimerization domain; TM, transmembrane; RAM, the RBP-Jκ-associated module; ANK, ankyrin repeats; TAD, transactivation domain; PEST, sequence rich in proline, glutamic acid, serine, and threonine. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Clinicopathological characteristics and NOTCH1 EGF-like repeats 10–13 mutations in Japanese OSCC.

Patient	T stage	N stage	Stage	Site	Histological differentiation	Survival	Mutation	Amino acid
2	4a	0	IV	Tongue	Well	Alive	G1133A	p.S378N
6	1	0	I	Tongue	Well	Alive	G1160A	p.C387Y
30	2	1	IV	Up gum	Well	Alive	G1181A	p.G394D
32	1	0	I	Low gum	Well	Alive	G1393A	p.A465T
39	4b	0	IV	Buccal	Well	Alive	G1393A	p.A465T
51	2	0	II	Tongue	Well	Alive	G1127A	p.C376Y
77	4a	2b	IV	Up gum	Moderate	Alive	G1174A	p.V392I
94	1	2b	IV	Palate	Well	Dead (pneumonia)	G1127A	p.C376Y

Abbreviations: T, tumor stage; N, lymph node metastasis stage.

42.0 months, $P < 0.01$), for patients with well differentiated tumors than for those with moderate or poor differentiated tumors (42.9 vs. 30.2 months, $P < 0.01$), for patients who were lymph node positive than for those who were lymph node negative (28.7 vs. 45.5 months, $P < 0.01$), and for patients with extracapsular spread lymph node metastasis than for those without (23.1 vs. 39.9 months, $P = 0.014$). Median OS was significantly shorter for patients who were lymph node positive than for those who were lymph node negative (33.4 vs. 43.6 months, $P < 0.01$), but did not differ with postoperative treatment, histological differentiation, lymph node metastasis and extracapsular spread metastatic lymph node did not differ significantly.

3.2. NOTCH1 mutations in Japanese OSCC

Sequencing of the entire *NOTCH1* coding region in 6 OSCC cell lines identified one point mutation, G1243A (p.E415K), in EGF-like repeat 11 in one cell line. In clinical samples, we examined NOTCH1 exon 6–9 corresponding to EGF-like repeats 10–13, which is ligand binding region. Point mutations were identified in eight (9.5%) of 84 tumors (Table 1 and Fig. 1) and all correspond to amino acid substitutions. No mutation was found in the histologically normal tissues that were resected along with *NOTCH1*-mutated tumors.

The found mutations were all nonsynonymous G > A transitions (Table 2 and Fig. 1). There were six types of mutations. Five types occurred in EGF-like repeat 10: one mutation at codon 376 (C > Y) in two tumors and one mutation at codon 378 (S > N), codon 387 (C > Y), codon 392 (V > I) and codon 394 (G > D), each in one tumor. One type occurred in EGF-like repeat 12 at codon 465 (A > T) in two tumors (Table 2). The rate of G > A transitions was 86.7% for EGF-like repeats 10–13, which was significantly higher than 45.2% for the entire area of NOTCH1 in HNSCC and OSCC according to the previously described data ($P < 0.001$) [5,8,23].

None of the above mutations was found in the Catalog Of Somatic Mutations In Cancer of Sanger Institute (Hinxton, UK) as of August 28th, 2014.

3.3. Survival outcome

At a median follow-up time of 27.0 months, 23 (27.4%) patients relapsed and 18 (21.4%) died in all 84 patients. The median and 2-year DFS were 38.0 months (95% CI, 32.0–40.3 months) and 71.3%, respectively. The median and 2-year OS were 41.7 months (95% CI, 34.6–42.7 months) and 79.0%, respectively.

Median DFS was significantly longer for patients with *NOTCH1*-mutated tumors than for those with *NOTCH1*-normal tumors (no relapse vs. 38.7 months, $P = 0.042$; Fig. 2). Median OS was not significantly different between the groups.

3.4. 3D structural simulation

X-ray diffraction data are available only for EGF-like repeats 11–13 of human NOTCH1 [21], and EGF-like repeat 12 is most

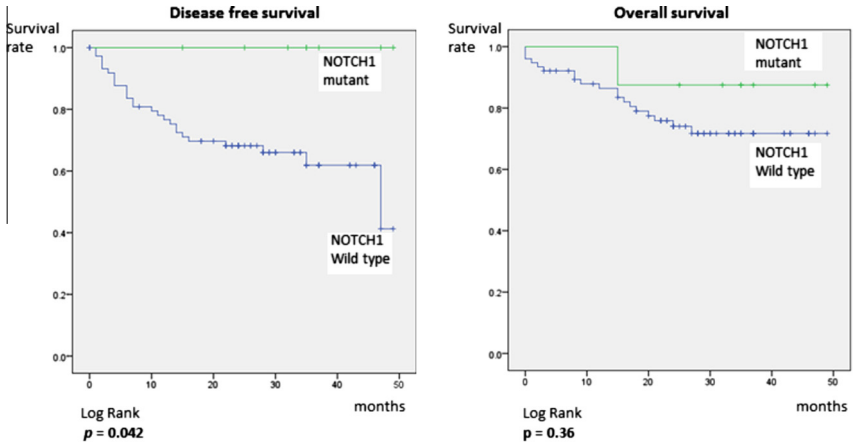

Fig. 2. Disease free and overall survivals of Japanese OSCC patients with or without NOTCH1 mutations. Kaplan–Meier curves for disease free survival (left panel) and overall survival (right panel) are shown.

critical for ligand binding [10–13]. Therefore, we chose p.A465T from Japanese OSCC and p.G481S from Caucasian [5], both in EGF-like repeat 12, for the 3D structural simulation. The p.A465T mutation would increase the solvent accessibility of the relevant amino acid residue, leading to a conformational change of NOTCH1 EGF-like repeats 11–13 (Fig. 3A and B). The p.G481S mutation would cause a loss of flexibility around this residue due to the newly formed hydrogen bond (Fig. 3C).

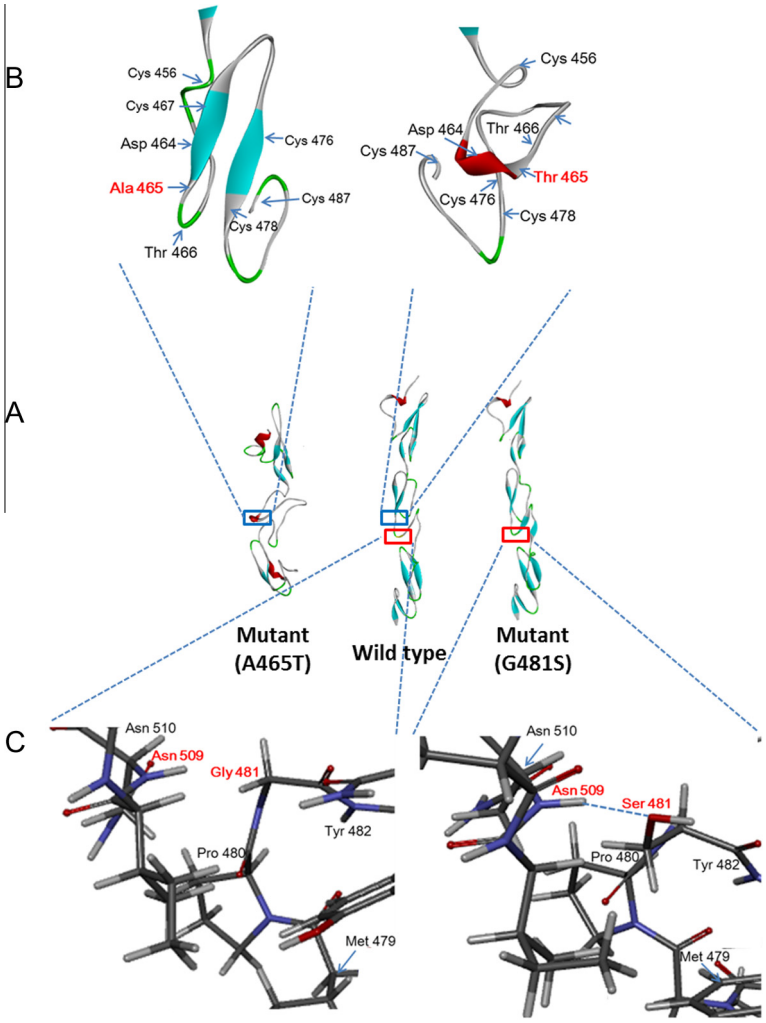

Fig. 3. Architecture of EGF-like repeats 11–13. (A) The overall structure of EGF-like repeats 11–13 is shown for wild-type, A465T mutant, and G481S mutant. (B) Enlarged images of EGF-like repeat 12. Red, blue and green ribbons represent α-helix, β-sheet and coils, respectively. (C) Enlarged images of the vicinity of mutation sites. Broken bars represent hydrogen bonds. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

It is established that NOTCH1 plays an important role in not only lymphomas and leukemia, but also various solid cancers [9,22–25]. Exome sequencing studies demonstrated that the frequency of *NOTCH1* mutation in HNSCC has a wide range of variation (11.8–15.6% in Caucasian HNSCC [4,5] or 16.0–43.1% in Asian OSCC [8,23]). These reports showed that most of the mutations in HNSCC including OSCC occurred in NOTCH1 NECD, and hypothesized that these mutations affect ligand binding. These findings are in marked contrast to TALL and chronic lymphocytic leukemia, where activating mutations of *NOTCH1* were present adjacent to the heterodimerization domain (HD), transactivation domain (TAD) and sequence rich in proline, glutamic acid, serine, and threonine (PEST) [15,24,25]. Our present study revealed a high frequency of mutations in NOTCH1 NECD and, thus in line with above hypothesis, suggests that ligand binding is compromised in Japanese OSCC.

The critical role of human NOTCH1 EGF-like repeats 11–12 in ligand binding is well established [11–13,26–28]. Recent studies suggested interactions within different NOTCH1 EGF-like repeats [29]. It was also reported that EGF-like repeat 10 may modulate ligand binding region through a direct steric effect [10]. However, the significance of these findings remains to be understood. We focused our study upon NOTCH1 EGF-like repeats 10–13 coding region. This region had point mutations in 9.5% of Japanese OSCC, a rate higher than that in Caucasian HNSCC (0–6.3%) [4–7], Indian OSCC (2.0%) [23] and Chinese OSCC (1.9%) [8]. Our analysis further showed that G > A transitions were significantly more frequent in EGF-like repeats 10–13 than the entire NOTCH1 in HNSCC and OSCC ($P < 0.001$) [5,8,23]. These data revealed a high incidence of mutations in NECD and suggest the likelihood of compromised NOTCH1 ligand binding in Japanese OSCC.

A recent study reported that NOTCH1 expression was downregulated in OSCC and concluded that downregulation of NOTCH1 expression plays a major role in the histopathogenesis of epithelial dysplasia [30]. In this regard, our 3D structural simulations indicated that the p.A465T mutation found in 2 Japanese well-differentiated OSCC would compromise ligand biding. Since altered NOTCH1 ligand biding function is likely to have effects similar to that of NOTCH1 downregulation, our present findings provide further support for the above notion.

Our data showed that Japanese OSCC patients with *NOTCH1*-mutated tumors had a longer median DFS as compared to *NOTCH1*-normal tumors. Although *NOTCH1* mutations were associated with a shorter DFS in Chinese OSCC [8], only one mutation of 42 *NOTCH1* mutations found in 51 Chinese OSCC was located in the ligand binding region. Thus, our present DFS is likely to represent the impact of mutations affecting NOTCH1 ligand binding. Since most of the previous reports concerned HNSCC rather than OSCC specifically, further studies on OSCC patients are necessary to unveil clinical significance of NOTCH1 mutations.

In conclusion, *NOTCH1* mutations in the vicinity of the ligand binding region occur frequently in Japanese OSCC. These mutations may affect ligand binding and impact on tumor progression and differentiation, and patient survival. Further studies are warranted to clarify the function of NOTCH signaling in OSCC.

Acknowledgments

We are grateful to Professor Naoya Nakamura and Dr. Yusuke Kondo, Department of Pathology, Tokai University School of Medicine, for providing pathological data, Professor Hiroyuki Kobayashi, Department of Clinical Pharmacology, Tokai University School of Medicine, for confirming statistic data, Dr. Toshio Homma for critical reading, and Tadayuki Sato, the Support Center for Medical Research and Education, Tokai University, for technical support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.021.

References

[1] S. Warnakulasuriya, Global epidemiology of oral and oropharyngeal cancer, Oral Oncol. 45 (2009) 309–316.
[2] R. Sankaranarayanan, Oral cancer in India: an epidemiologic and clinical review, Oral Surg. Oral Med. Oral Pathol. 69 (1990) 325–330.
[3] K.B. Jadhav, N. Gupta, Clinicopathological prognostic implicators of oral squamous cell carcinoma: need to understand and revise, N. Am. J. Med. Sci. 5 (2013) 671–679.
[4] N. Agrawal, M.J. Frederick, C.R. Pickering, C. Bettegowda, K. Chang, R.J. Li, et al., Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1, Science 333 (2011) 1154–1157.
[5] N. Stransky, A.M. Egloff, A.D. Tward, A.D. Kostic, K. Cibulskis, A. Sivachenko, et al., The mutational landscape of head and neck squamous cell carcinoma, Science 333 (2011) 1157–1160.
[6] W. Sun, D.A. Gaykalova, M.F. Ochs, E. Mambo, D. Arnaoutakis, Y. Liu, et al., Activation of the NOTCH pathway in head and neck cancer, Cancer Res. 74 (2014) 1091–1104.
[7] D.A. Gaykalova, E. Mambo, A. Choudhary, J. Houghton, K. Buddavarapu, T. Sanford, et al., Novel insight into mutational landscape of head and neck squamous cell carcinoma, PLoS ONE 9 (2014) e93102.
[8] X. Song, R. Xia, J. Li, Z. Long, H. Ren, W.T. Chen, et al., Common and complex Notch1 mutations in Chinese oral squamous cell carcinoma, Clin. Cancer Res. 20 (2013) 701–710.
[9] P. Ranganathan, K.L. Weaver, A.J. Capobianco, Notch signalling in solid tumours: a little bit of everything but not all the time, Nat. Rev. Cancer 11 (2011) 338–351.
[10] J. Cordle, C. Redfieldz, M. Stacey, P.A. van der Merwe, A.C. Willis, B.R. Champion, et al., Localization of the delta-like-1-binding site in human Notch-1 and its modulation by calcium affinity, J. Biol. Chem. 283 (2008) 11785–11793.
[11] I. Rebay, R.J. Fleming, R.G. Fehon, L. Cherbas, P. Cherbas, S. Artavanis-Tsakonas, Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor, Cell 67 (1991) 687–699.
[12] A. Sharma, A.N. Paranjape, A. Rangarajan, R.R. Dighe, A monoclonal antibody against human Notch1 ligand-binding domain depletes subpopulation of putative breast cancer stem-like cells, Mol. Cancer Ther. 11 (2012) 77–86.
[13] S. Hambleton, N.V. Valeyev, A. Muranyi, V. Knott, J.M. Werner, A.J. McMichael, et al., Structural and functional properties of the human notch-1 ligand binding region, Structure 12 (2004) 2173–2183.
[14] R.L. Davis, D.L. Turner, Vertebrate hairy and enhancer of split related proteins: transcriptional repressors regulating cellular differentiation and embryonic patterning, Oncogene 20 (2001) 8342–8357.
[15] A.P. Weng, A.A. Ferrando, W. Lee, J.Pt. Morris, L.B. Silverman, C. Sanchez-Irizarry, et al., Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia, Science 306 (2004) 269–271.
[16] F. Radtke, K. Raj, The role of Notch in tumorigenesis: oncogene or tumour suppressor?, Nat Rev. Cancer 3 (2003) 756–767.
[17] L.W. Ellisen, J. Bird, D.C. West, A.L. Soreng, T.C. Reynolds, S.D. Smith, et al., TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms, Cell 66 (1991) 649–661.
[18] E.J. Allenspach, I. Maillard, J.C. Aster, W.S. Pear, Notch signaling in cancer, Cancer Biol. Ther. 1 (2002) 466–476.
[19] K. Sakamoto, T. Fujii, H. Kawachi, Y. Miki, K. Omura, K. Morita, et al., Reduction of NOTCH1 expression pertains to maturation abnormalities of keratinocytes in squamous neoplasms, Lab. Invest. 92 (2012) 688–702.
[20] H. Hijioka, T. Setoguchi, A. Miyawaki, H. Gao, T. Ishida, S. Komiya, et al., Upregulation of Notch pathway molecules in oral squamous cell carcinoma, Int. J. Oncol. 36 (2010) 817–822.
[21] J. Cordle, S. Johnson, J.Z. Tay, P. Roversi, M.B. Wilkin, B.H. de Madrid, et al., A conserved face of the Jagged/Serrate DSL domain is involved in Notch trans-activation and cis-inhibition, Nat. Struct. Mol. Biol. 15 (2008) 849–857.
[22] N. Agrawal, Y. Jiao, C. Bettegowda, S.M. Hutfless, Y. Wang, S. David, et al., Comparative genomic analysis of esophageal adenocarcinoma and squamous cell carcinoma, Cancer Discovery 2 (2012) 899–905.
[23] India Project Team of the International Cancer Genome Consortium, Mutational landscape of gingivo-buccal oral squamous cell carcinoma reveals new recurrently-mutated genes and molecular subgroups, Nat. Commun. 4 (2013) 2873.
[24] X.S. Puente, M. Pinyol, V. Quesada, L. Conde, G.R. Ordonez, N. Villamor, et al., Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia, Nature 475 (2011) 101–105.

[25] M.B. Mansur, R. Hassan, T.C. Barbosa, A. Splendore, P.Y. Jotta, J.A. Yunes, et al., Impact of complex NOTCH1 mutations on survival in paediatric T-cell leukaemia, BMC Cancer 12 (2012) 9.
[26] W.R. Gordon, K.L. Arnett, S.C. Blacklow, The molecular logic of Notch signaling–a structural and biochemical perspective, J. Cell Sci. 121 (2008) 3109–3119.
[27] C. Ge, T. Liu, X. Hou, P. Stanley, In vivo consequences of deleting EGF repeats 8–12 including the ligand binding domain of mouse Notch1, BMC Dev. Biol. 8 (2008) 48.
[28] S.J. Bray, Notch signalling: a simple pathway becomes complex, Nat. Rev. Mol. Cell Biol. 7 (2006) 678–689.
[29] A. Sharma, A. Rangarajan, R.R. Dighe, Antibodies against the extracellular domain of human Notch1 receptor reveal the critical role of epidermal-growth-factor-like repeats 25–26 in ligand binding and receptor activation, Biochem. J. 449 (2013) 519–530.
[30] R. Yoshida, M. Nagata, H. Nakayama, K. Niimori-Kita, W. Hassan, T. Tanaka, et al., The pathological significance of Notch1 in oral squamous cell carcinoma, Lab. Invest. 93 (2013) 1068–1081.