Establishment and characterization of a clear cell odontogenic carcinoma cell line with EWSR1-ATF1 fusion gene

Satoko Kujiraoka, Takaaki Tsunematsumi, Yukiko Sato, Maki Yoshida, Ayataka Ishikawa, Rei Tohyama, Michio Tanaka, Yutaka Kobayashi, Tomoyuki Kondo, Aya Ushio, Kunihiro Otsuka, Mie Kurosawa, Masako Saito, Akiko Yamada, Rieko Arakaki, Hirokazu Nagai, Hiromasa Nikai, Kengo Takeuchi, Toshitaka Nagao, Youji Miyamoto, Naozumi Ishimaru, Yutaka Kobayashi, Tomoyuki Kondo, Yasusei Kudo

Abstract

Objective: Clear cell odontogenic carcinoma (CCOC) is a rare malignant odontogenic tumor (MOT) characterized by sheets and lobules of vacuolated and clear cells. To understand the biology of CCOC, we established a new cell line, CCOC-T, with EWSR1-ATF1 fusion gene from a mandible tumor with distant metastasis and characterized this cell line.

Materials and methods: To detect the EWSR1-ATF1 fusion gene, we used three CCOC cases, including the present case, by RT-PCR and FISH analysis. We characterized established CCOC-T cells by checking cell growth, invasion and the expression of odontogenic factors and bone-related factors. Moreover, the gene expression profile of CCOC-T cells was examined by microarray analysis.

Results: Histologically, the primary tumor was comprised of cords and nests containing clear and squamoid cells separated by fibrous septa. In addition, ameloblastomatous islands with palisaded peripheral cells were observed, indicating probable odontogenic origin. This tumor expressed the fusion gene EWSR1-ATF1, which underlies the etiology of hyalinizing clear cell carcinoma (HCCC) and potentially that of CCOC. We found a breakpoint in the EWSR1-ATF1 fusion to be the same as that reported in HCCC. Established CCOC-T cells grew extremely slowly, but the cells showed highly invasive activity. Moreover, CCOC-T cells expressed bone-related molecules, odontogenic factors, and epithelial mesenchymal transition (EMT)-related molecules.

Conclusion: To the best of our knowledge, this is the first report on the establishment of a CCOC cell line. CCOC-T cells serve as a useful in vitro model for understanding the pathogenesis and nature of MOT.

Article history:
Received 20 January 2017
Received in revised form 10 March 2017
Accepted 5 April 2017
Available online 10 April 2017

Keywords:
Clear cell odontogenic carcinoma
Malignant odontogenic tumor
Hyalinizing clear cell carcinoma
EWSR1-ATF1 fusion genes

Introduction

A malignant odontogenic tumor (MOT) is a rare tumor that comprises up to 2.1% of all odontogenic tumors [1]. Among them, clear cell odontogenic carcinoma (CCOC) is quite a rare MOT. To date, 81 cases have been reported in the literature [2–14].Histologically, CCOC commonly shows a biphasic tumor pattern consisting of large sheets of clear epithelial cells and irregular cords of basoloid cells with scant eosinophilic cytoplasm, supported by a fibrous stroma [15]. As clear cell areas are frequently seen in other oral neoplasms, it is important to rule out these neoplasms of the oral and maxillofacial regions to obtain a correct diagnosis.

Recently, Bilodeau et al. identified an EWSR1 rearrangement in five out of six CCOC cases, with confirmation of ATF1 involvement in one case [16,17]. In addition, a case of CCOC with EWSR1 and ATF1 rearrangement has been reported [11]. However, the break-
point of EWSR1-ATF1 fusion has not been clarified in CCOC. Hyalinizing clear-cell carcinoma (HCCC) is the first epithelial neoplasm shown to be associated with EWSR1-ATF1 fusion [18]. HCCC is a rare minor salivary gland carcinoma composed of clear cells forming cords and nests in a hyalinized stroma [19]. As CCOC and HCCC have extensive morphologic and immunohistochemical overlap [20], these two types of tumor are difficult to distinguish histologically despite a difference in cell origin and location. These findings suggest that either CCOC represents an intraosseous HCCC or that CCOC represents an “odontogenic analogue” to HCCC.

To know the biology and pathogenesis of CCOC, we established a CCOC cell line with EWSR1-ATF1 fusion and characterized this cell line.

Materials and methods

Tissue samples

A tissue sample obtained from a CCOC of a 64-year-old Japanese woman was used for generating a cell line. This study was approved by the Ethic Committee of Tokushima University (#1924) and was performed in compliance with the Declaration of Helsinki. At the time of tissue sampling, we informed the patient of the use of the tissues for future research and the patient complied. Informed consent was obtained verbally.

To detect the EWSR1-ATF1 fusion gene, we used three CCOC cases including the present case (obtained from Tokushima University Hospital and Tokyo Metropolitan Hiroo Hospital), one HCCC case (obtained from Tokyo Medical University Hospital) and one oral squamous cell carcinoma (OSCC) case (obtained from Tokushima University Hospital). Formalin-fixed paraffin-embedded (FFPE) samples were used after obtaining approval from the ethics committee of each institution. Informed consent from all patients was obtained both verbally, as well as written, through a signature from all patients in this study.

Immunohistochemistry (IHC)

For the IHC analysis of original tumor tissues, paraffin-embedded sections were deparaffinized and subsequently applied to heat-induced antigen retrieval in 10 mM citrate buffer (pH 6.0). The sections were incubated with primary antibody, subsequently developed using horseradish peroxidase conjugated anti-rabbit immunoglobulin (Vector Laboratories), and counterstained with hematoxylin. The antibodies used in this study are listed in Supplementary Table 1.

Fluorescence in situ hybridization (FISH)

FISH analysis of fusion genes was performed with DNA probes for EWSR1 and ATF1. Unstained sections (4 µm thick) were subjected to hybridization with the EWSR1 break-apart probe kit (Vysis LSI EWSR1 dual-color, break-apart rearrangement probe; Abbott Molecular) with bacterial artificial chromosome clone-derived probes for ATF1 (CTD-2174I21). Hybridized slides were then stained with DAPI and examined using a BX51 fluorescence microscope (Olympus).

RT-PCR

Total RNA was obtained from FFPE samples using NucleoSpin® total RNA FFPE (Macherey-Nagel) and from the cultured cells using RNeasy Mini kits (Qiagen). The cDNA was synthesized from 1 µg total RNA using a PrimeScript RT reagent kit (Takara Bio). The primers used for RT-PCR analysis are listed in Supplementary Table 2. For detecting EWSR1-ATF1 fusion gene, aliquots of the total cDNA were amplified with Tks Gflex™ DNA Polymerase (Takara Bio), and amplification was performed in a T100™ thermal cycler (Bio-rad) for 40 cycles following an initial denaturation at 98 °C for 10 s and 62 °C for 30 s. For conventional RT-PCR, aliquots of the total cDNA were amplified with Go Taq® Green Master Mix (Promega), and amplification was performed in a T100™ thermal cycler for 35 cycles after an initial denaturation at 95 °C for 30 s, an annealing at 60 °C for 30 s, and an extension at 72 °C for 1 min.

Cell culture

The tissue sample obtained from a CCOC was cut into small pieces and placed into 90-mm dishes (Falcon) with DMEM (Sigma-Aldrich) containing 10% fetal bovine serum (FBS) (Gibco). When these outgrowth cultures formed a confluent monolayer, the cells were subcultured after enzymatic removal with 0.05% trypsin-EDTA for the first passage. Then, we subcultured the cells for over 100 passages and used for the following analyses. A mucoepidermoid carcinoma (MEC) cell line, NCI-H292, was purchased from American Type Culture Collection and maintained in RPMI-1640 (Sigma-Aldrich) supplemented with 10% FBS. The OSCC cell line, HOC313, and an immortalized odontogenic epithelial cell line, iOdE, were maintained in DMEM supplemented with 10% FBS. The immortalized ameloblastoma cell line, AM-1, and the immortalized gingival epithelial cell line, OBA-9, were maintained in Keratinocyte-SFM (Invitrogen).

Cytogenetic analysis

Chromosomes were stained with Hoechst 33258. Images were captured using the Axio Imager 2 (Zeiss). The karyotypes of at least eight well-banded metaphases were analyzed.

Electron microscopic analysis

The cells were fixed with 2% of paraformaldehyde and 2% glutaraldehyde (GA) in 0.1 M phosphate buffer (PB) pH 7.4. Then, they were fixed with 2% GA in 0.1 M PB at 4 °C overnight. After fixation, the samples were postfixed in 2% osmium tetroxide with 0.1 M PB at 4 °C for 1 h. The samples were then dehydrated in graded ethanol, followed by dehydration in graded ethanol solutions. Then, the samples were transferred to a resin (Quetol-812, Nissin EM Co., Tokyo, Japan) and polymerized at 60 °C for 48 h. The polymerized resins were ultra-thin sectioned at 70 nm with a diamond knife using an ultramicrotome and the sections were mounted on copper grids. The ultrathin sections were double-stained with uranyl acetate and lead citrate. The grids were observed by a transmission electron microscope (JEM-1400Plus, JEOL Ltd., Tokyo, Japan) at an acceleration voltage of 80 kV. Digital images (2048 x 2048 pixels) were taken with a CCD camera (VELETA, Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Xenograft

CCOC-T cells (1 x 10^6) were resuspended in 50 µL of Cellmatrix type I-A (Nitta Gelatin, Osaka, Japan), which is an acid-soluble collagen isolated from the tendon of a pig. Cells were injected subcutaneously into multiple sites of male SCID mice (CREA Japan Inc., Tokyo, Japan). This study was conducted in accordance with the “Fundamental Guidelines for the Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions” under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government. This study’s protocol was approved by the Committee on Animal Experiments at Tokushima University (#15011). The animals were monitored.
for the formation of a tumor mass every week and were sacrificed two months later.

In vitro invasion assay

Invasiveness was assessed by an invasion assay using a 24-well cell culture insert with 8 μm pores (Falcon). The filter was coated with 20 μg of Matrigel (Becton Dickinson), a reconstituted basement membrane substance. The lower compartment contained 0.5 mL of medium. Following trypsinization, 1.2 × 10^5 cells were resuspended in 100 μL of DMEM supplemented with 10% FBS and placed in the upper compartment of the cell culture insert for 23 h. At the end of the incubation, the cells were fixed with methanol and stained with hematoxylin. The cells on the upper surface of the filter were removed by wiping with a cotton swab and the invasiveness of the cells was determined by counting via a light microscope at 100× magnification. We performed the assay three times and three fields were randomly selected for counting for each assay.

Microarray analysis

The total RNA from the CCOC-T and NCI-H292 cells was isolated using an RNeasy Mini kit (Qiagen). The RNA quality was first checked for chemical purity using a NanoDrop spectrophotometer and then assessed for RNA integrity using the Bioanalyzer 2100 (Agilent Technologies). The total RNA (100 ng) was amplified and labeled using the Affymetrix Whole-Transcript (WT) Sense Target Labeling Protocol, and the labeled RNA was hybridized to a GeneChip Human Gene 2.0 ST Array (Affymetrix). Gene expression data for the iOdE and OBA-9 cells was previously obtained [21–24]. Data visualization and analysis was performed using GeneSpring GX (Version 12.1) software. The accession number for the microarray dataset reported in this paper is GSE84300.

Results

CCOC case

A 64-year-old Japanese woman was referred for oral surgery for a radiolucent lesion. Panoramic radiography revealed an irregular-shaped and partially well-defined radiolucent lesion ranging from the lower left cuspid to the right retromolar region (Fig. 1A). A CT scan revealed an osteolytic lesion with a moth-eaten appearance of the lower left cuspid to the right retromolar region (Fig. 1A). A CT scan revealed an osteolytic lesion with a moth-eaten appearance combined with the disappearance of cortical bone (Fig. 1B). Fluorodeoxyglucose (18F)-PET (FDG-PET) showed abnormal uptake at the sixth right rib and pubis. With a provisional diagnosis of MOT, surgical resection of the mandibular tumor was performed. The gross examination of the resected specimen revealed a white tissue mass (Fig. 1C). After surgery, treatment was completed with chemotherapy. For the metastatic lesion of the rib and pubis, radiation therapy was performed. However, approximately one year after the surgery, other metastatic lesions were found in the left clavicle and right scapula.

Histology of CCOC case

The microscopic examination of this case revealed an epithelial tumor in the mandible consisting of small and irregular sheets or cords of neoplastic cells separated by dense collagenous stroma (Fig. 1D and E). The tumor islands were primarily composed of clear and squamoid cells (Fig. 1D and E). The clear cells contained various amounts of diastase-digestable PAS-positive granules and showed no positivity for alcin blue, indicating the glycogen content of the cytoplasm (Supplementary Table 3). In some areas, the tumor cells formed a tubular pattern (Fig. 1F). Moreover, the tumor cells were round to polygonal, had a clear cytoplasm, and exhibited cellular atypism. The IHC analysis showed that the tumor cells expressed cytokeratin AE1/AE3, p63, and CK-19 (Fig. 2A–C). As the differential diagnosis of a jaw tumor with clear cells, CCOC, intraosseous salivary gland tumors (e.g., MEC, epithelial-myoepithelial carcinoma, myoepithelial carcinoma, and HCCC), melanotic tumors, metastatic renal cell carcinoma and the clear-cell variant of calcifying epithelial odontogenic tumor were raised [21–24]. Tumor cells were negative for S100, vimentin, EMA, GFAP, αSMA, HMB-45, UCHL-1, CD10 and CD31 (Supplementary Table 3) and did not contain amyloid and mucus-producing cells (data not shown), indicating that the above-mentioned tumors, except for CCOC and HCCC, were ruled out. As CCOC and HCCC exhibit a morphologic and immunophenotypic overlap [24,25], it was difficult to distinguish CCOC and intraosseous HCCC. In this tumor, however, odontogenic features, such as the presence of ameloblastomatous islands with palisaded peripheral cells and the positivity of CK-19, were observed (Fig. 2C and D). Finally, this tumor was diagnosed as CCOC.

In this tumor, the Ki-67 labeling index was over 30% (Fig. 2E). Furthermore, neural invasion and necrosis were also observed (Fig. 2F). Clinically there was metastasis to the sixth right rib, pubis, left clavicle, and right scapula (Fig. 2G). These findings indicated a high degree of malignancy. Interestingly, this tumor strongly expressed EGFR, which can be a useful target of anticancer therapy (Fig. 2H).

EWSR1-ATF1 fusion gene in cases of CCOC

We performed two-color FISH for EWSR1, and ATF1 genes in our case. Our case showed rearrangement of both EWSR1 and ATF1 genes (Fig. 3A). Then, we confirmed EWSR1-ATF1 fusion transcripts by RT-PCR from an FFPE sample of this tumor (Fig. 3B). The direct sequence of the EWSR1-ATF1 fusion transcript revealed that exon 11 of EWSR1 was fused to exon 3 of ATF1 (Fig. 3B), indicating that the breakpoint is the same as that found in HCCC [18]. Moreover, we examined the detection of EWSR1-ATF1 fusion transcripts by RT-PCR from FFPE samples of two other CCOC cases (Case #1 and #2). An OSCC case (Case #4) was used as a negative control. Information and histology regarding the cases used in this experiment is presented in Fig. 3C, Supplementary Tables 4 and 5. The EWSR1-ATF1 fusion transcript was detected in all two CCOC cases (Fig. 3B). As expected, the EWSR1-ATF1 fusion transcript was also detected in the HCCC case (Case #3), but not in the OSCC case. Interestingly, we found that Case #1–3 had the same breakpoint of the EWSR1-ATF1 fusion transcript as observed in our case (Fig. 3D and Supplementary Table 4).

Establishment of a CCOC cell line

CCOC-T cells were established from the above-mentioned CCOC case. We subcultured the cells to over P100. CCOC-T cells were spontaneously immortalized through bypassing senescence without any treatment and exhibited a polygonal shape (Fig. 4A). We performed a cytogenetic analysis by specifically examining the conventional karyotype of this cell line. The karyotypes of the CCOC-T cells were 50, XX, +5, +6, +8, +9, der(9;15)(q10;q10), del(12)(p12), −13, −16, +add(16)(q24)x2, −22, +mar1, and +mar2 (Fig. 4B). Among twenty cells examined in this analysis, the number of chromosomes was between 48 and 50 (Fig. 4C).

Upon EM examination of the CCOC-T cells, the tumor cells exhibited features of squamous cell differentiation, such as desmosomes with the insertion of tonofilaments (Fig. 4D). Moreover, the tumor cells demonstrated features of ductal cell differentiation, such as microvilli formation and intercellular or
intracytoplasmic lumina formation (Fig. 4E and F). Taken together, these findings are consistent with the histological features in the primary tumor.

CCOC-T cells were then injected subcutaneously with collagen gel into SCID mice to determine the level of tumor formation in vivo. After 2 month, tumor was observed, but size was small. The tumor nests were composed of both squamoid and clear cells (Fig. 4G). Tumor cells expressed p63, but not S100 (Supplementary Fig. S1). This result indicates that the tumors formed by the CCOC-T cells were histologically similar to the primary tumor.
Characterization of the CCOC cell line

We next characterized the CCOC-T cells. First, we examined the growth of the CCOC-T cells. Besides odontogenic carcinoma, MEC and OSCC also arise in the jaw as an intraosseous type. Therefore, we used NCI-H292 (MEC) and HOC313 (OSCC) cells in this experiment. NCI-H292 cells contain the CRTC1-MAML2 fusion gene, as previously reported (Fig. 5A) [26], and HOC313 cells are highly invasive cancer cells with EMT features [27]. The growth of CCOC-T cells was extremely slow, in comparison with the HOC313 and NCI-H292 cells (Fig. 5B). Next, we examined the invasion activity of these cells. The CCOC-T cells demonstrated signifi-
significantly higher invasive activity than the NCI-H292 cells (Fig. 5C and D). HOC313 cells revealed the highest invasive activity among all cell types examined.

We examined gene expression in the CCOC-T cells. We confirmed the expression of the EWSR1-ATF1 fusion gene in the CCOC-T cells (Fig. 5A). Then, we examined the expression of bone-related genes and odontogenic factors including enamel proteins in CCOC-T cells by RT-PCR. CCOC-T cells expressed RUNX2, BSP, COL1, OCN and MSX2 (Fig. 5E). NCI-H292 cells expressed only RUNX2. CCOC-T cells expressed amelogenin, amelotin and KLK4, which are involved in the generation of teeth (Fig. 5F). AM-1 cells were derived from ameloblastoma, which is a typical odontogenic tumor [28]. As expected, AM-1 cells expressed enamel proteins including amelogenin and amelotin (Fig. 5F).

As CCOC-T cells expressed odontogenic factors, such as amelogenin, amelotin and KLK4, we examined the expression of these molecules by RT-PCR in FFPE samples of CCOC cases (Cases #1 and #2), and a HCCC case (Case #3) as used in Fig. 2B. Although amelotin expression was not observed in all cases (data not shown), amelogenin expression was observed in one of CCOC cases (Case #2) (Fig. 5G). Moreover, KLK4 expression was observed in all CCOC cases but not in HCCC (Fig. 5G). These findings suggest that amelogenin and KLK4 may be used to distinguish between CCOC and HCCC as an odontogenic marker.

Gene expression profile of the CCOC cell line

To know the specific gene expression in CCOC, we compared the gene expression profile of CCOC-T and NCI-H292 cells using a microarray analysis. A total of 326 genes were found to be upregulated by more than fivefold, while 123 genes were downregulated by more than fivefold in CCOC-T cells compared with those of the NCI-H292 cells (Fig. 6A, Supplementary Table 6 and 7). CXCL14, MMP1, MMP2, MMP8, MMP12, ADAM28, FGF9, S100A7, PTCH1, CDH11, SNAI2, and N-cadherin were upregulated in CCOC-T cells. The upregulation of these genes was confirmed by RT-PCR (Fig. 6B). SNAI2 and N-cadherin are well known EMT-promoting factors. Therefore, we examined the expression of EMT-related genes, including SNAI1, E-cadherin and vimentin in CCOC-T cells. While the upregulation of SNAI1 and N-cadherin was observed, E-
cadherin downregulation and the upregulation of vimentin were not observed (Fig. 6B). Several invasion and EMT-related genes were also found to be upregulated (GSE84300).

Moreover, we compared the gene expression profile of CCOC-T, NCI-H292, OBA-9 and iOdE. iOdE cells are odontogenic epithelial cells established from epithelial cell rests of Malassez [29]. and OBA-9 is immortalized gingival epithelial cells. Among them, 183 genes were found to be commonly upregulated in the cell types of odontogenic origin, CCOC-T and iOdE cells (Fig. 6C and Supplementary Table 8).
Discussion

Here, the EWSR1-ATF1 fusion transcript was detected in both CCOC and HCCC cases. Interestingly, exon 11 of EWSR1 was fused to exon 3 of ATF1 in all CCOC cases, indicating the same breakpoint found in HCCC. Therefore, the EWSR1-ATF1 fusion gene as well as histological findings cannot be a distinguishing factor for CCOC and intraosseous HCCC. Cumulating evidences show that primary intraosseous salivary gland tumors are extremely rare. However, two cases of salivary clear cell carcinomas in the mandible which was distinguished from CCOC by the pattern of histological findings were reported. Several hypotheses have been proposed to explain the pathogenesis of intraosseous salivary gland tumors. Although one theory suggests that they may arise from ectopic

![Image](https://example.com/image.png)
salivary gland tissue that was developmentally entrapped within the jaw, the discovery of ectopic salivary gland tissue is extremely rare in biopsy specimens from the jaw [31]. Therefore, this seems unlikely source for intraosseous salivary gland tumors. The most likely source for intraosseous tumors is odontogenic epithelium. Indeed, mucous-producing cells and glandular structure are frequently found in the lining epithelium of odontogenic cysts. In our case, ameloblastomatous islands with palisaded peripheral cells were observed, suggesting the evidence of an odontogenic origin. These findings suggest that it is difficult to understand that a rare salivary gland carcinoma, HCCC develops in the jaw. Therefore, CCOC represents an “odontogenic analogue” to HCC. In addition, the expression of odontogenic factors and bone-related genes were observed in CCOC-T cells. Our microarray analyses revealed that 183 genes are commonly upregulated in CCOC-T cells and immortalized odontogenic epithelial cells among other type of cells including gingival epithelial cells and salivary gland tumor cells. This finding may help to identify the specific marker for CCOC. Moreover, we examined the karyotypes of the CCOC-T cells and found that the CCOC-T cells have a translocation (9;15)(q10;q10) and deletion of chromosome 12, suggesting that a specific abnormality may exist in CCOC. However, to identify the specific factors...
of CCOC for a differential diagnosis, further experiments are required.

The CCOC-T cells demonstrated enhanced invasiveness and the expression of several MMPs and EMT-promoting factors. This phenotype is consistent with the finding that the primary tumor exhibited malignant behaviors, such as perineural invasion and distant metastasis. Similar to other malignant tumors, EMT may play an important role in the malignant behaviors of CCOC. Interestingly, this tumor strongly expressed EGFR (Fig. 2H). Although there is no report on EGFR expression in odontogenic carcinoma, EGFR inhibitors may be used for the chemotherapy against odontogenic carcinoma including CCOC.

In summary, we established a CCOC cell line (CCOC-T) that exhibited the presence of the EWSR1-ATF1 fusion gene, odontogenic features, and highly invasive activity. This is the first report regarding the establishment of a CCOC cell line. We believe that CCOC-T cells can be a useful cellular model for understanding the pathogenesis and nature of MOT including CCOC. Moreover, CCOC-T cells may facilitate the discovery of diagnostic makers for the differential diagnosis of this type of cancer.

Conflict of interest statement

All contributing authors declare no conflicts of interest.

Acknowledgements

This study was supported by a Grant-in-Aid from the Ministry of Education, Science, and Culture of Japan. Technical assistance was provided from the Support Center for Advanced Medical Sciences (Tokushima University). The authors thank Mr. Hideaki Horikawa (Tokushima University) for technical assistance and Dr. Ikuo Ogawa (Hiroshima University) for valuable discussion. The authors also thank Dr. Shigehiro Abe and Dr. Naoko Yokomizo (Hiroo hospital) for providing clinical information. In addition, the authors thank Dr. Nobuyuki Kamata (Hiroshima University), Dr. Harada (Iwate Medical University), and Dr. Shinya Murakami (Hiroo hospital) for providing clinical information. In addition, the authors thank Dr. Nobuyuki Kamata (Hiroshima University), Dr. Harada (Iwate Medical University), and Dr. Shinya Murakami (Osaka University) for kindly providing HOC313, AM-1, and OBA-9 cells, respectively.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.oraloncology.2017.04.003.

References