The assessment of oral squamous cell carcinoma

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DOI:
10.33612/diss.135865241

CHAPTER 8

Detection of tumor cells in saliva from patients with oral squamous cell carcinoma using DNA hypermethylation of *KCNA5* and *TIMP3*

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Manuscript submitted

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ABSTRACT

Background: The high local recurrence and/or second primary tumor rate of 20-30% in patients with oral squamous cell carcinoma (OSCC) is partly caused by residual tumor cells of the first primary tumor and the presence of precancerous epithelium that has not (yet) clinically manifested. Since OSCC cells are shed into the oral cavity, the detection of tumor-specific DNA methylation markers in saliva could be a tool for the early detection of local recurrences or second primary tumors of OSCC. The aim of this explorative study was to identify and validate new methylation markers to detect OSCC cells in saliva. For that purpose, we investigated molecular biomarkers methylated in OSCC and not in normal cells from a genome-wide methylation screening analysis using MethylCap-Seq analysis of 12 OSCCs compared to controls. In addition, we selected 4 markers reported to be methylated in saliva by others (*EDNRB, HOXA9, NID2* and *TIMP3)*.

Results: Using our OSCC methylome, seven genomic locations representing six genes (*C11orf85*, *CMTM2*, *FERMT3*, *KCNA5*, *SIPA1* and *TBX4*) were identified that were significantly hypermethylated in tissues of OSCC compared to DNA from controls. QMSP analysis using saliva from OSCC patients compared to non-cancer controls of similar age or younger age, revealed only a difference for *KCNA5* methylation (respectively $p = 0.003$ and $p = 0.001$). Moreover, when *KCNA5* was combined with other markers, the combination with *TIMP3* revealed a 100% diagnostic potential in detecting OSCC patients compared to non-cancer controls of similar age using saliva.

Conclusions: In this explorative study we identified several new OSCC-specific methylation markers with a high sensitivity and high negative predictive value for the detection of OSCC in saliva. Two methylation markers (*KCNA5* and *TIMP3*) might be useful for early detection of local recurrence or second primary tumors in saliva cells of OSCC patients. A larger prospective study should be done to confirm the clinical relevance of these two markers.

BACKGROUND

Oral Squamous Cell Carcinoma (OSCC) is the most common subtype of head and neck cancer. It is the sixth most common cancer worldwide, accounting for 650,000 new cases and 350,000 related deaths annually [1]. Over the last 30 years, the incidence of OSCC has almost doubled, while the 5-year survival increased by 10% [2], reaching a 5-year survival of only 48% [3]. Risk factors for recurrence of OSCC are locally residual cancer after treatment of the first primary tumor or field cancerization of the oral mucosa [4,5].

Residual tumor cells are isolated cells of the first primary tumor which can remain after treatment and have the potential to develop into a local recurrence. Due to the small size of isolated cells and often submerged location, these residual tumor cells are often discovered late by regular clinical examination or imaging [5].

Due to the long-term exposure to tobacco and alcohol the epithelium of the upper aerodigestive tract might harbor areas with accumulation of pre-cancerous (epi)genetic changes [6,7], with or without clinical manifestation which is known as field cancerization [5]. These (epi)genetic changes drive carcinogenesis (6) and therefore areas with field cancerization are at risk of developing a new malignant tumor [8].

Besides the difficulty in detecting residual tumor cells/precancerous epithelial cells and the challenge of detecting the conversion of clinical visible precancerous fields (e.g. leukoplakia and erythroplakia) into new tumors as early as possible, the detection of local recurrences at an early stage is complicated by the consequences of earlier treatment. The resection area of the first primary tumor might be reconstructed with tissue from extra-oral donor sites and fibrosis is induced by surgery and irradiation [9]. Although local recurrences and new primary tumors are clinically difficult to detect at an early stage, (epi)genetic alterations in DNA from residual primary tumor cells or field cancerization cells released into saliva might be detectable before clinical manifestation of recurrent disease [10]. Using (epi)genetic alterations to detect tumor DNA in saliva is therefore a promising new non-invasive strategy for the early detection of local recurrences.

Alteration in DNA methylation status is one of the epigenetic aberrations that drives tumor genesis in OSCC [11]. Changes in DNA methylation are associated with etiological factors such as cigarette smoking and alcohol consumption [6,7] through regulation of DNA methyltransferases (DNMT) [8,12,13]. Changes in DNMT expression might result in genome-wide hypermethylation associated with one of the hallmarks of cancer, chromosomal instability [14] as well as the downregulation of tumor suppressor genes [14]. Moreover, DNA methylation changes occurs early in tumorigenesis [14]. Therefore, DNA methylation

markers might also be useful for the early detection of tumor cells or be detectable in shed DNA fragments in liquid biopsies such as plasma and sputum [10] and has been reported in lung [15], breast [16], colorectal [16] and hepatocellular cancer [17]. The detection of tumor cells in saliva of patients with head and neck SCC has been reported as well [18-20] and requires markers with high sensitivity and high specificity. In patients with OSCC, only few markers that are methylated in tumor tissue but not in normal epithelium, have been reported [10]. To identify new methylation markers in patients with OSCC that are associated with lymph node status, we recently used a genome-wide methylation screening method based on MethylCap-Seq analysis [21] and reported a methylome of several OSCC cases and numerous new differentially methylated tumor markers [22,23].

In the current study, to identify new biomarkers associated with OSCC and not with other tissue, we assessed the available methylome of a series of OSCC cases generated by MethylCap-Seq analysis [23] with the methylome of 80 control tissues. We describe the identification of several new markers which are significantly hypermethylated in OSCC and not in non-cancer control samples. We validated the performance of these OSCC specific DNA hypermethylation markers using quantitative methylation specific PCR (QMSP) in a proof of principle pilot study with saliva of OSCC patients and non-cancer controls. In addition, we included five DNA methylation markers previously reported to be associated with OSCC[19,20,24]. The aim of this study was to identify methylation markers with a high sensitivity and a high negative predictive value (NPV) for the detection of tumor cells in saliva from patients with OSCC.

MATERIALS AND METHODS

Identification of novel methylation markers using MethylCap-seq analysis

The strategy of methylation marker selection is summarized in Figure 1. To identify genomic loci hypermethylated in OSCC and not in normal tissue, *in silico* analysis was performed of MethylCap-Seq data [25] as reported previously [21,26]. In summary, 12 OSCC samples and two pools of leukocytes of 500 ng DNA each were fragmented using Covaris S2 (Covaris, Woburn, MA, USA). Subsequently, methylated DNA fragments were separated from unmethylated fragments by enrichment with the MethylCap kit (Diagenode, Belgium), paired-end sequenced using the Illumina Genome Analyzer II and mapped to the human reference genome (NCBI build 37.3). For further analysis, only pair-end sequenced fragments (reads) were included that could be mapped to unique specific loci, and summarized using an in house generated . "Map of the Human Methylome" for MethylCap-seq data [27].

For further analyses only the MCs that are located either in a promoter region, between 2000 bp upstream to 500 bp downstream of the Transcription Start Site (TSS) or in the first exon of an Ensemble (v65), gene were selected and statistically compared using R with R-package Bayseq [28]. The most equally methylated MCs amongst all 12 OSCC were ranked according the likelihood of equal methylation. Additionally, an approximate false discovery rate (FDR) was calculated. The 5000 most equally methylated MCs with the lowest FDR were used for further analysis. These highest ranked 5000 MCs in OSCC were compared to the 2276 MCs available in the MethylCap-Seq data of the two leukocyte pools, by the Mann-Whitney U test (wilcox.test function in R). All MCs with a p-value <0.05 were selected for further analyses (n = 335, Supplementary data 1). In the next step, all MCs were selected with a 100% positive and negative predictive value defined by ≤2 reads in both leukocytes pools as well as ≥3 reads in all 12 OSCC (Supplementary data 1). Finally, the MCs were compared to the semi-quantitative methylation data of the "Map of the Human Methylome" (non-OSCC primary tumor samples (n = 32), non-OSCC cancer stem cells (n = 11), normal tissue (n = 22), stem cells (n = 6) and normal cell lines (n = 9)) [27] to select MCs without methylation detected in the average methylome.

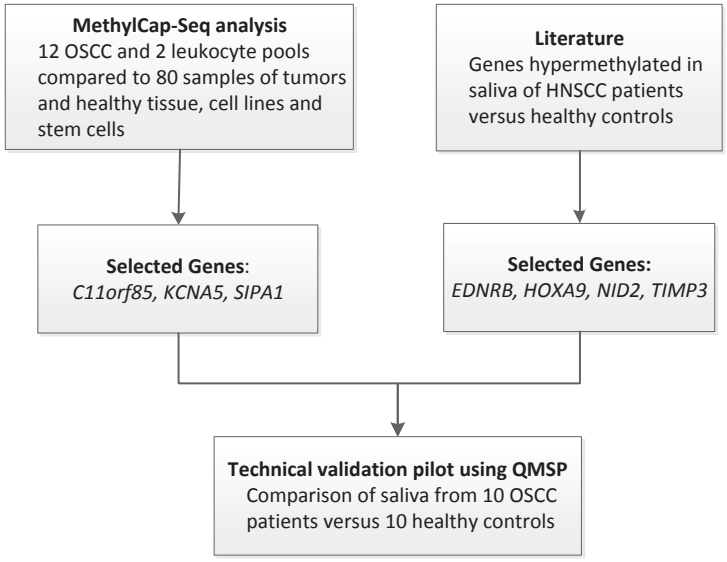


Figure 1. Study design. Methylation markers were selected using a MethylCap-Seq protocol. Selected genes were technically validated in a pilot study with saliva from 10 OSCC patients and 10 healthy controls (five younger and age-matched controls) and compared to methylation markers associated with OSCC and selected from literature.

Abbreviations: OSCC, oral squamous cell carcinoma; HNSCC, head and neck squamous cell carcinoma; QMSP, quantitative methylation-specific PCR.

Technical validation of OSCC-methylation markers

For the validation, saliva from in total 10 OSCC patients were collected: seven males and three females with a median age of 63 years and with pT1-2 (n = 7) and pT3-4 (n = 3) staged tumors. For methylation status in the original tumor tissue, six fresh frozen (FF) tumor biopsies and nine formalin fixed paraffin embedded (FFPE) tumor resection tissues were available for DNA isolation. Saliva samples were collected from healthy (non-cancer) controls. Five patients were planned to undergo benign corrective jaw surgery (median age 45 years, significant younger than the OSCC patients p = 0.050) and five patients were scheduled to receive dental implants (median age 67 years, age-matched with the OSCC patients). Characteristics of the patients and controls are summarized in Table 1. All patients and controls had no prior history of HNSCC or immunological diseases such as Sjögren's syndrome and no apparent infections in the oral cavity during saliva collection. Saliva was collected preoperatively on the day of surgery between 07:00 and 10:00 AM to exclude variation due to circadian rhythm. Patients and controls had at least 90 min without stimulation of the salivary glands by drinking, smoking or eating. Patients and controls deposited 2 ml whole saliva into a 15 ml falcon tube without a time limit. Samples were recoded for lab processing.

Ethics approval and consent to participate

Written approval and informed consent of all twenty patients and controls included in the validation study was obtained. Because of the non-invasive character of saliva sample collection, this research was not a clinical study with human subjects as meant in the Medical Research Involving Human Subjects Act as was concluded by the local Medical Ethics Review Board of the University Medical Center Groningen (M12.116657) and no further approval was required.

DNA isolation

Saliva DNA integrity was preserved by adding 2.5 ml of 1 tablet Roche Complete mini Protease Inhibitor Cocktail (pro. #. 04693159001) dissolved in 10 ml filtered (4°C) PBS. The saliva PBS mixture was equally divided in three 1.5 ml Eppendorf Tubes and centrifuged at 14000 rpm for 10 min at 4°C. The pellets were incubated in 600 µl 1% SDS-proteinase K. Both the pellet and the supernatant were separately stored at -80°C.

Tumor DNA was isolated as follows. Approximately eight 10 µm thick sections were cut from the FFPE blocks. For quality control, the first and last section (3 µm thick) were HE-stained to check for tumor load. A dedicated head and neck pathologist marked areas with >60% neoplastic cells. The 10 µm FFPE sections were deparaffinized using xylene and

neoplastic-enriched areas were macrodissected and used for DNA extraction. From the FF tissues, approximately four 10 µm thick sections were cut. Both, the FF and FFPE sections were incubated overnight at 60°C in 300 µl 1% SDS-proteinase K solution.

Table 1. Clinical characteristics of all included subjects

Patient characteristics	OSCC Patients (n)	Non age-matched Controls (n)	Age-matched Controls (n)
Total	10	5	5
Age (years) *			
Median (IQR)	63 (58 to 74)	45 (30 to 62)	67 (57 to 70)
Gender **			
Male	7	2	4
Female	3	3	1
Saliva DNA yield (µg)***			
Median (range)	64 (6 to 140)	32 (16 to 75)	32 (20 to 57)
FFPE tumor tissue	9	NA	NA
FF tumor tissue	6	NA	NA
Tumor localization		NA	NA
Tongue	4	NA	NA
Gum	2	NA	NA
Floor-of-mouth	3	NA	NA
Cheek	1	NA	NA
pT		NA	NA
1-2	7	NA	NA
3-4	3	NA	NA
pN		NA	NA
0	5	NA	NA
+	2	NA	NA
X	3	NA	NA
Infiltration depth (mm)		NA	NA
Median (range)	3 (1 to 23)	NA	NA
Tumor diameter (mm)		NA	NA
Median (range)	22 (7 to 52)	NA	NA

* OSCC versus orthognatic, p = 0.050; no significant differences between the other groups . ** No significant differences between patient and control groups. *** No significant differences between patient and control groups. Abbreviations: OSCC, oral squamous cell carcinoma; IQR, interquartile range; ug, microgram; mm, millimeter; NA, not applicable; FFPE, formalin fixed, paraffin embedded; FF, fresh frozen.

DNA was extracted from sections and saliva cell pellets by phenol-chloroform extraction and ethanol precipitation as described previously [29]. Samples were dissolved in TE-4 buffer (50 µl for FFPE and FF, 300 µl for saliva) and stored at 4°C. DNA quality and quantity was assessed using the Nanodrop and Biomed II PCR protocol [30].

Bisulfite treatment and Quantitative Methylation Specific PCR (qMSP)

Isolated DNA was treated with bisulfite for methylation-specific-PCR (MSP) as previously described [23,29]. Briefly, bisulfite treated DNA (bisDNA) was acquired using the EZ DNA methylation kit (Zymogen, BaseClear, Leiden, The Netherlands), according to the manufacturer's protocol. Methylation-specific-PCR (MSP) was performed on 20 ng bisDNA as follows: 10 min 95°C, 40 cycli (1 min 95°C, 1 min $T_{annealing}$, 1 min 72°C), followed 10 min 72°C and ∞ 4°C. Primer sequences and $T_{annealing}$ are summarized in Table 2. As controls in each qMSP, leukocyte DNA from healthy individuals (as a control for endogenous methylation), leukocyte DNA that was *in vitro* methylated (I.V.) by SssI methyltransferase (New England BioLabs Inc., Bioké, Leiden, The Netherlands) (as a control for methylated DNA) and leukocyte DNA that was amplified according to manufacturer's protocol using whole genome amplification with the Illustra Ready-To-Go GenomiPhi HY DNA Amplification Kit (GE Healthcare, Little Chalfont, UK) (as a control for hypomethylation). Cytosine conversion by bisulfite treatment was checked with primers specific for bisulfite treated Beta-Actin (ACTB) and DAPK as described earlier [31,32]. After MSP, PCR products were separated and visualized by custom Ethidium Bromide staining.

QMSP was performed as previously described with an internal dual-labeled hybridization probe (IDT, Coralville, IA) [29,31]. For *CMTM2* and *FERMT3* no specific primers and probes within 250 bp of the methyl core region could be designed. For four genes (*C11orf85*, *KCNA5*, *SIPA1* and *TBX4*), QMSP primers and probes were designed by Methyl Primer Express TM Software v1.0 (Thermo Fisher Scientific, Applied Biosystems, Leiden, The Netherlands) and checked using Clone Manager software (Sci-Ed software, Denver, USA) (Table 2). Serial dilutions of I.V. DNA were used to calculate standard curves for each primer-probe set, resulting in suitable conditions for the detection of methylation of *C11orf85, KCNA5* and *SIPA1*. For *TBX4* no optimal condition was found and therefore *TBX4* was excluded for further analysis. The amount of bisulfite treated DNA input of each sample was determined by qMSP for ACTB (Table 2) as reported previously [31]. Fluorescence was measured in triplicates for 50 cycles using the following mixture: 7.5 µl of 2* LightCycler 480 Probes Master mix (Roche Diagnostics GmbH, Mannheim), 300 nM of forward and reverse primers (IDT, Coralville, IA), 200 nM of probe (IDT) and 2.5 µl bisulfite-modified DNA (~25 ng). Each sample was analyzed by LightCycler 480 (Roche Diagnostics GmbH, Mannheim). Relative methylation

Table 2. The sequences of the primers and probes for all genes used for methylation detection by QMSP and MSP

Gene name	Method	QMSP forward 5'-3'	QMSP reverse 5'-3'	QMSP probe 6-FAM 5'-3'TAMRA	Amplicon length	$T_{annealing}$	Reference
ACTB	QMSP	TGGTGATGGAGGAGGTTTAGTAAGT	AACCAATAAAACCTACTCCTCCCTTAA	ACCACCACCCAACACACAATAACAAACACA	133	60	NA
C11orf85	QMSP	GAAATGCGTACGCGTAGATC	CAACTTCGAAACTCGTACCG	TGGGAAGCGTATTTGCGCGTGC	118	60	NA, MethylCap-Seq
EDNRB	QMSP	GGGAGTTGTAGTTTAGTTAGTTAGGGAGTAG	CCCGCGATTAAACTCGAAAA	TTTTTATTCGTCGGGAGGAG	75	60	Demokan et al.[19]
HOXA9	QMSP	AATAAATTTTATCGTAGAGCGGTAC	CATATAACAACTTAATAACACCGAA	GCGCCCCCATTAACCGTACGCGT	226	60	Guerrero-Preston et al.[24]
NID2	QMSP	GCGGTTTTTAAGGAGTTTTATTTTC	CTACGAAATTCCCTTTACGCT	ACGCCGCTACCCCAAACCTTACGA	99	62	Guerrero-Preston et al.[24]
KCNA5	QMSP	TTTTTTGACGTTAGGGTTAAGC	GAACGCCTAACGTCAAACTC	AGAGGGGTCGGTCGATCGTTGG	103	60	NA, MethylCap-Seq
SIPA1	QMSP	TTCGAGTCGAGGTTAGTTC	CAAATCGACTAACCTCTTCG	CGTAGCGGTAGCGATGTAGGC	124	60	NA, MethylCap-Seq
TBX4	QMSP	TTCGTTTTTAGTTCGAGTTGC	CTACGCTCTCAATCCTACGC	CGGCGTTAGTGGACGCGG	99	60	NA, MethylCap-Seq
TIMP3	QMSP	GCGTCGGAGGTTAAGGTTGTT	CTCTCCAAAATTACCGTACGCG	AACTCGCTCGCCCGCCGAA	95	62	Sun et al.(19)
ACTB	MSP	TAGGGAGTATATAGGTTGGGGAAGTT	AACACACAATAACAAACACAAATTCAC		103	57	Melchers et al.[29]
DAPK meth	MSP	GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCGA		98	60	Melchers et al.[29]
DAPK unmeth	MSP	GGAGGATAGTTGGATTGAGTTAATGTT	CCCTCCCAAACACCAACC		101	60	Melchers et al.[29]

Abbreviations: NA, not applicable; (Q)MSP, (quantitative) methylation specific pcr; (un)meth, (un)methylated.

levels for each sample were calculated as ratios using absolute measurements: the average DNA quantity of the gene of interest divided by the average DNA quantity of *ACTB* and then multiplied by 10,000.

The PubMed electronical database was searched for DNA methylation biomarkers that were reported to be hypermethylated in saliva of head and neck SCC patients compared to saliva of healthy controls. This search revealed four genes, *EDNRB* [19], *HOXA9* [24], *NID2* [24] and *TIMP3* [20] which were used as a reference. QMSP primers and probes were selected from literature for *EDNRB, HOXA9, NID2* and *TIMP3* [19,20,24] (Table 2).

Statistical analysis

The Mann-Whitney U test was used for comparing MethylCap-Seq read counts of OSCC and leukocytes and was also used for comparing methylation levels between saliva of patients and controls. Optimal cut-offs were determined by ROC-curves. The diagnostic potential of the biomarkers in detecting OSCC in saliva was determined by the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). Differences in methylation levels between the tumors and saliva within the OSCC patients were compared using the related Wilcoxon signed-rank test. All tests were performed two-tailed. Results were considered significant when $p < 0.05$ or FDR < 0.05. Statistical analysis was performed with IBM SPSS Statistics 23 (Statistical Package for the Social Sciences, Inc., Chicago, IL, USA).

RESULTS

Selection of OSCC specific methylation markers

With the MethylCap-Seq analysis, a total of 11.6 to 22.3 x 10^6 reads were sequenced per sample. Approximately 6.91 to 14.6 x 10^6 unique reads could be mapped back to the genome per sample [22,23]. Statistical analysis of reads around the transcription start site resulted in a ranking list of the 5000 most significant equally methylated regions among the 12 OSCCs. In total 335 methylation cores (MCs) representing 319 genes were significantly differentially methylated between the 12 OSCC samples and the two leukocyte pools (Supplementary data 1). Of these 335 MCs, 53 MCs were not hypermethylated in the leukocytes (≤2 reads). Seven of these MCs were hypermethylated (≥3 reads) in all OSCC and had a 100% positive and negative predictive value for hypermethylation in OSCC and leukocytes. These seven MCs were associated with six genes: *C11orf85*, *CMTM2*, *FERMT3*, *KCNA5*, *SIPA1* and *TBX4*. Semi-quantitative comparison with the methylation data in the Map of the Human Methylome showed no methylation in a panel of 80 reference samples that were considered as samples not associated with OSCC (thus considered as negative controls). For *TBX4*, *CMTM2* and *FERMT3* no suitable QMSP primers/probes could be designed or (Q)MSP did not pass the

technical validation. The design and technical validation of *C11orf85*, *KCNA5* and *SIPA1* QMSP was optimal for further analysis. In addition, based on literature search we included *EDNRB* [19], *HOXA9* [24], *NID2* [24] and *TIMP3* [20], as these genes were reported to be associated with OSCC in saliva.

Technical validation of OSCC-specific methylation markers to detect tumor cells in saliva from patients with OSCC

To select methylation markers with high sensitivity and high specificity, we collected a total of 2 ml of saliva from 10 patients with OSCC and from 10 non-cancer controls (five orthognathic and five dental implant patients) referred to as controls in this study (Table 1). Median amount of isolated DNA from saliva was 64 µg (range: 6 to 140 µg) among the OSCC patients, 32 µg (range: 16 to 75 µg) among the orthognathic patients and 32 µg (range: 20 to 57 µg) among the dental implant patients (Table 1). There were no significant differences in DNA yield from the pellets between the OSCC, orthognathic and dental implant patients.

QMSP analysis of the seven selected methylation markers on bisulfite-treated DNA from saliva cells from 10 OSCC patients and 10 controls, revealed significant differences in methylation levels of *EDNRB* ($p = 0.016$) and *KCNA5* ($p < 0.001$) (Figure 2). In fact, methylation of *C11orf85*, *HOXA9, NID2* and *SIPA1* was detected in all controls and methylation of *EDNRB* in 50% of the controls (not associated with age) (Figure 2). Five control patients were significantly younger than the OSCC patients (Table 1). Comparing QMSP data from saliva from OSCC with either controls of similar or younger age, revealed a difference for only *KCNA5* methylation (both OSCC-patients vs younger or older controls $p = 0.001$) and *EDNRB* methylation (only OSCC vs younger controls $p = 0.003$) (data not shown). Age-matched analysis did not affect the results of the other methylation markers.

One explanation for the fact that not all markers were hypermethylated in saliva cells of OSCC patients compared to saliva cells of controls could be that the original tumor is not methylated for each of these methylation markers. To evaluate the effect on the sensitivity and NPV of detecting tumor cells in saliva in patients with methylated tumor tissues, the methylation status of these seven markers was tested in available tumor tissues of these same 10 OSCC patients. Methylation of four markers (*EDNRB, C11orf85, KCNA5* and *SIPA1)* was detected in all 10 tumor tissues (Supplementary data 2). Methylation was detected in nine (*HOXA9*) and seven (*NID2* and *TIMP3*) of the 10 tumor tissues (Supplementary data 2). When performing the analysis with OSCC cases showing methylation of tumor tissue of *HOXA9, NID2* or *TIMP3*, no differences were found in methylation levels in saliva between OSCC cases and controls (data not shown).

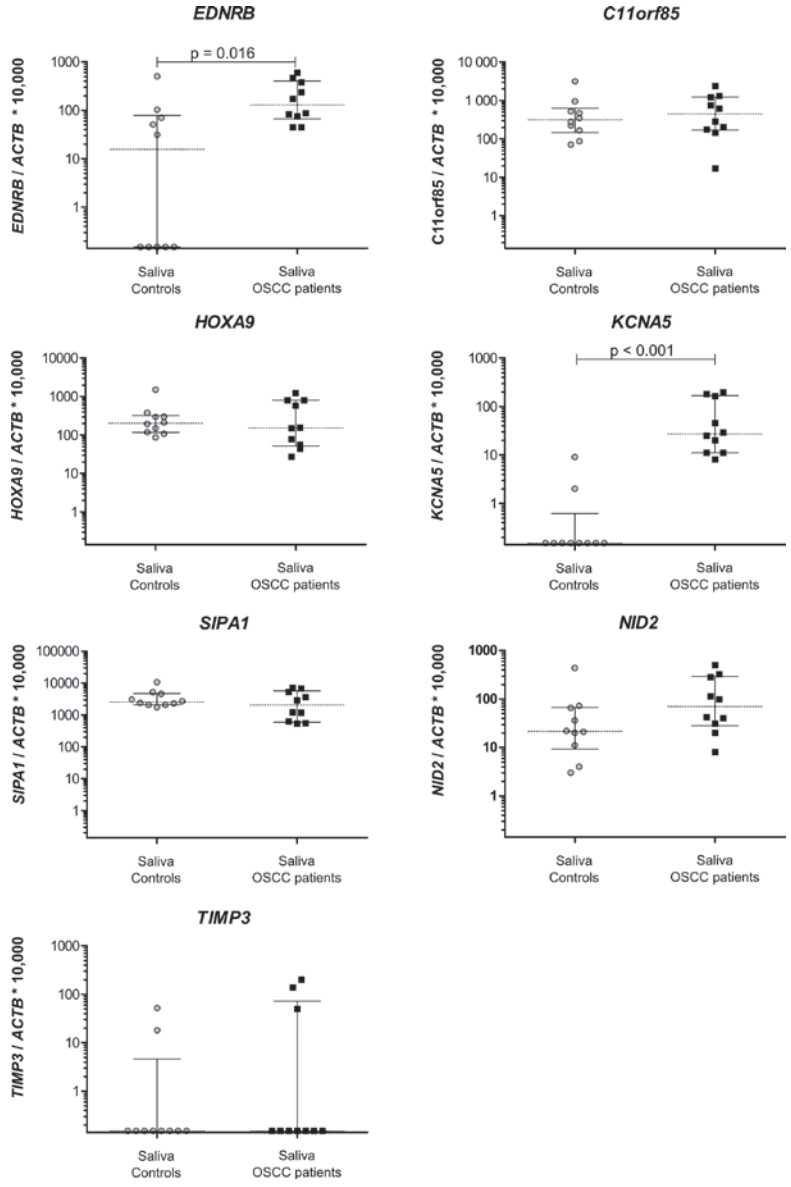


Figure 2. DNA methylation levels of seven OSCC-specific markers in saliva cells of OSCC patients and healthy controls. QMSP analysis of seven methylation markers using DNA extracted from cells in saliva collected from 10 OSCC patients (saliva OSCC patients) and as healthy control saliva from five younger and five age-matched controls (saliva controls). Methylation levels on the x-axis are defined as the average DNA quantity of the gene of interest divided by the average DNA quantity of *ACTB* and then multiplied by 10,000. Dotted and continuous line represents median with interquartile range. Only statistically significant differences ($p < 0.050$, using the Mann-Whitney-U test) between saliva of 10 controls and 10 OSCC samples are shown.

Table 3. OSCC diagnostic potential in saliva of the selected methylation markers

	A. OSCC patients (n = 10) vs all controls (n = 10)						B. OSCC patients (n = 10) vs age-matched controls (n = 5)					
Gene name	**AUC**	**Optimal cut-off**	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**	**AUC**	**Optimal cut-off**	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**
C11orf85	0.56	559	50	80	71	62	0.44	533	50	60	71	38
EDNRB	0.82	38	100	60	71	100	0.68	22	100	40	77	100
HOXA9	0.44	479	40	90	80	60	0.39	479	40	80	80	40
KCNA5	0.99	5	100	80	83	100	0.98	10	90	100	100	83
NID2	0.72	27	80	60	67	75	0.66	38	70	80	88	57
SIPA1	0.40	5239	20	90	67	53	0.32	5239	30	80	75	36
TIMP3	0.57	34	30	90	75	56	0.65	25	30	100	100	42

A ROC analysis of methylation in saliva between 10 OSCC patients and 10 control patients (A) and an age-matched analysis of saliva between 10 OSCC patients and five dental implant control patients (B) for the optimal cut-off points to detect OSCC in saliva. *KCNA5* combined with *TIMP3* could detect OSCC with a 100% sensitivity, specificity, PPV and NPV in an age-matched analysis**.**
Abbreviations: AUC, area under the curve; PPV, positive predictive value; negative predictive value; OSCC, oral squamous cell carcinoma.

To evaluate the possible clinical relevance for the detection of tumor cells in saliva independent on methylation status in the original OSCC tissue, we determined the optimal cut-off to discriminate between OSCC and non-cancer control DNA in saliva cells for each marker. ROC analysis among all 20 patients (10 OSCC patients versus 10 controls), revealed a high area under the curve (AUC) with a 100% sensitivity and 100% NPV of *EDNRB* (AUC 0.82) and *KCNA5* (AUC 0.99) for detecting patients with OSCC using saliva cells (Table 3A). The other five markers showed a lower sensitivity and NPV when using the most optimal cut-off. The analysis on the age-matched patients (10 OSCC versus five aged-matched controls) resulting in other optimal cut-offs also showed a 100% sensitivity and 100% NPV for *EDNRB* (AUC 0.68) (Table 3B). For *KCNA5* (AUC 0.98) both the sensitivity (90%) and NPV (83%) decreased slightly, but interestingly with the highest specificity (100%) and positive predictive value (PPV 100%) (Table 3B). As none of the markers had a 100% diagnostic potential, we combined one or more methylation markers in age matched samples. This analysis revealed that *KCNA5* combined with *TIMP3* had the highest diagnostic potential (100% for this limited dataset) in detecting saliva cells in patients with OSCC (data not shown).

DISCUSSION

DNA methylation of OSCC specific tumor markers might be useful as biomarkers for early detection of new primaries or local recurrences in OSCC patients, preferably prior to clinical manifestation. In this study we used the methylome of tissue biopsies of 12 patients with OSCC generated using genome-wide methylation screening by MethCapSeq analysis [23] to identify DNA methylation biomarkers with a high diagnostic potential for the detection of OSCC. Seven new OSCC-specific biomarkers representing six genes were identified by selection of equally methylated markers between all 12 OSCC and not methylated in two pools with leukocytes from four different individuals. Moreover, the acquired highest ranking methylated candidate markers were compared to a vast methylome database of over 80 different samples considered as negative control samples. For the validation of these markers using QMSP, we could design optimal primers/probes assays for three markers (*C11orf85, KCNA5* and *SIPA1*). To evaluate biomarkers with the highest performance, DNA was isolated from saliva cells acquired from 10 OSCC patients and their corresponding tumor tissues. Saliva cells from five younger controls and five age-matched controls planned to undergo benign surgery served as healthy (non-cancer) controls. *KCNA5* was the best marker (independent of age) as it was significantly hypermethylated in OSCC saliva cells in comparison to control saliva. The possible clinical relevance of *KCNA5* is further illustrated by the very high sensitivity (90%), NPV (83%), specificity (100%) and PPV (100%), the highest of all markers tested in this study (Table 3B). Moreover, a panel of *KCNA5* and *TIMP3* could

further improve the diagnostic potential of detecting OSCC in saliva cells (100%) in an age matched analysis. Due to the limited size of our pilot group, the diagnostic potential of these biomarkers must be validated on a larger independent and prospective cohort. Similarly, a saliva database containing samples of 5-year-follow-up, pre- and post-operative as well as pre-malignant cases should be constructed for prospective studies and to assess the background methylation caused by non-tumor cells.

The use of molecular markers for the early detection and monitoring treatment response and disease progression using body fluids like saliva, sputum, plasma, cerebrospinal fluid and urine [10,33] has limited clinical utility today [34] but has great promise to contribute to improved clinical care by early detection of OSCC or monitoring the treatment response. Since DNA methylation is important in carcinogenesis, occurs early in tumorigenesis and is detectable in patient saliva [35], DNA methylation markers could contribute to the early detection of local recurrences of OSCC. Additionally, aberrations in DNA methylation arise early in tumorigenesis [14]. Therefore, our results warrants further analysis in larger independent cohorts.

Several methylation markers for the detection of cells in saliva of patients with OSCC were reported previous (*EDNRB, HOXA9, NID2* and *TIMP3*) [19,20,24]. As a comparison to our new markers, we analyzed these markers in parallel on the same samples using QMSP. In our cohort, methylation of *HOXA9* and *NID2* was detected in all saliva cells of health individuals. Methylation of *EDNRB* was observed in 50% of these saliva, but the difference between saliva of OSCC patients and of age-matched controls was not significant. An explanation for the frequent methylation in normal control, especially in the saliva of the "older" age-matched "healthy" (non-cancer) saliva cells is that methylation of many genomic sequences has been reported to increase with age [36]. Therefore, methylation of these reported genes are not suitable as methylation markers in the "older" age-matched OSCC cohort.

Note that the four markers selected from literature showed significant methylation in our age-matched samples from normal saliva, which can also be an explanation why these markers were not present in our selected list of 2276 highest ranking methylation cores from the MethylCap-seq analysis of OSCC tissue samples.

With the genome-wide methylation analysis, within the methylome of millions of methylated DNA fragments in 12 OSCC samples, we eventually identified and validated three of the six new candidate markers for OSCC *(C11orf85, KCNA5* and *SIPA1)*. The pathophysiology of the novel genes related to OSCC or other types of cancer is not yet fully clarified. *KCNA5* is a member of the voltage-gated potassium (K_V) channel subfamily A [37]. In Ewing sarcoma cells methylation of the *KCNA5* promoter region is correlated with cell survival and

proliferation [38]. Signal-induced proliferation associated protein 1 (*SIPA1*) is located at the 11q13 chromosome close to *CCND1* (cyclin D1) and is known for influencing growth factors and cytokines by regulating *RAP1* in hematopoietic cells [39,40]. Loss of *SIPA1* resulted in myeloproliferative disorders in mice [40]. The interaction between *SIPA1* and *RAP1* is also associated with metastasis in breast and prostate cancer by mediating cell adhesion signaling and metastasis suppressor gene signaling [40]. Recently, *SIPA1* was found to be overexpressed in OSCC and correlated to lymph node metastasis [41]. *C11orf85* also called *MAJIN* (membrane anchored junction protein) plays a role in telomere attachment to the inner nuclear membrane during meiosis [42,43]. *C11orf85/MAJIN* is related to cancer as one of the genes in a 92-gene signature that is prognostic for overall survival in multiple myeloma patients [44]. Currently, no studies are available that report the exact role of *C11orf85/MAJIN* in oncogenesis. The biological significance of these three methylated genes in OSCC has not been elucidated in great detail and needs further investigation in future.

CONCLUSIONS

In conclusion, using the methylome of 12 OSCC tissue samples based on a genome-wide methylation screening approach, we have identified several novel biomarkers commonly methylated in OSCC. With one of these methylation markers (*KCNA5*) cells in saliva that are associated with OSCC patients could be detected with a high diagnostic potential. Moreover, it is of interest to perform a larger scale evaluation for *KCNA5* combined with *TIMP3, given the* 100% diagnostic potential found for detecting OSCC cells in saliva. Irrespective of the small study size, our findings demonstrate the high sensitivity of Quantitative Methylation Specific PCR for detecting methylation on saliva cell DNA. DNA methylation detection using saliva has potential as an easy, low-cost, non-invasive and accurate diagnostic tool to improve the early detection of local recurrences or second primary tumors in OSCC. Our findings warrant evaluation of the clinical relevance of these methylation markers in larger cohorts.

SUPPLEMENTARY DATA

Supplementary data 1. OSCC hypermethylation markers selected with MethylCap-Seq data. All 335 Methylation Cores (MCs) with a p-value < 0.05 between the 5000 highest ranked MCs in OSCC compared to the 2276 MCs available in the MethylCap-Seq data of the two leukocyte pools by Mann-Whitney-U using R and the wilcox.test function. The final seven MCs representing six genes with a 100% positive and negative predictive value defined by ≤2 reads in both leukocytes pools as well as ≥3 reads in all 12 OSCC are highlighted in bold and underlined.

					Number of reads													
Gene name	**Chromosome locus**	**Gene regio start (GSE42409)**	**Gene regio end (GSE42409)**	**p-value (Mann-Whitney U test)**	**OSCC patient 1**	**OSCC patient 2**	**OSCC patient 3**	**OSCC patient 4**	**OSCC patient 5**	**OSCC patient 6**	**OSCC patient 7**	**OSCC patient 8**	**OSCC patient 9**	**OSCC patient 10**	**OSCC patient 11**	**OSCC patient 12**	**Leukocyte pool 1**	**Leukocyte pool 2**
NUDT14	14	105649604	105650313	0.000	7	9	10	8	8	5	6	9	11	6	10	9	14	14
AC012074.3	2	25595094	25595647	0.000	9	9	9	5	9	10	6	13	9	8	10	10	14	14
PIGQ	16	614601	615239	0.000	5	9	9	11	2	4	8	5	9	9	14	7	15	15
AC021021.1	2	6635378	6635815	0.000	8	6	7	8	6	6	14	9	10	9	12	8	14	14
KCNA5	12	5153088	5153505	0.000	6	4	11	6	5	10	12	11	7	6	10	3	1	1
RAPSN	11	47470539	47471210	0.000	7	7	8	12	7	10	8	14	7	10	11	8	14	14
RP11-56M3.1	10	92913015	92913355	0.000	9	10	7	10	5	7	7	13	9	8	10	9	13	13
KIF22	16	29800874	29801501	0.000	3	8	5	8	6	9	11	5	6	10	9	7	12	12
AC007272.7	2	201963822	201964264	0.000	4	7	7	6	6	4	7	9	6	6	10	6	10	10
AL359844.1	10	70782804	70783197	0.000	5	12	10	6	4	6	9	13	9	6	11	8	14	14
NAT12	14	57855731	57856072	0.000	8	9	9	6	5	6	7	12	8	10	8	6	4	4
EIF2S2	20	32702015	32702335	0.000	6	10	8	6	9	9	14	15	8	9	11	4	3	3
ACTBP11	1	224052444	224052660	0.000	6	10	9	4	5	11	9	13	12	4	14	1	17	16
PTPRS	19	5341427	5341949	0.000	7	9	5	7	5	5	14	5	7	7	8	8	12	12
AC017104.4	2	232254143	232254467	0.000	5	12	9	10	3	11	12	11	10	5	13	7	15	15
TBX4	17	59531961	59532563	0.000	5	13	7	5	4	9	13	9	11	4	12	4	1	0
PABPCP2	2	147344801	147345449	0.000	8	13	9	10	9	4	13	15	8	9	10	7	15	15
AC113607.1	2	905373	905826	0.000	3	10	17	10	10	5	11	18	16	3	10	10	20	19
TH	11	2192576	2193202	0.000	5	11	6	12	8	2	7	15	4	5	13	5	15	15
SIPA1	11	65408027	65408751	0.000	6	9	13	7	4	10	16	9	14	3	9	7	2	1
GPR39	2	133174634	133175088	0.000	9	14	8	5	4	5	17	5	9	4	17	5	17	17
ING5	2	242665670	242666172	0.000	3	10	2	9	6	8	13	9	7	5	13	5	14	15
C11orf85	11	64739412	64739716	0.000	9	8	9	6	4	7	15	11	8	4	11	3	2	2
AC011530.1	19	46318069	46318747	0.000	6	10	12	9	9	3	13	11	10	3	11	5	3	3
AL139130.1	1	156357691	156358065	0.000	11	8	8	3	1	8	16	10	13	4	8	4	1	0
MUC2	11	1075204	1076015	0.000	4	13	7	7	7	10	8	17	7	8	17	10	16	16
SLC22A20	11	64981227	64981938	0.000	4	8	10	9	8	9	17	12	7	5	13	4	3	3
AP001476.3	21	47457079	47457365	0.000	3	10	6	9	5	7	13	11	7	6	16	4	14	15
C3orf24	3	10149532	10150224	0.000	5	10	7	8	6	11	10	10	15	6	10	7	13	13
AL031296.2	1	12587915	12588225	0.000	7	11	7	5	5	5	12	11	4	3	11	6	12	12
CENPB	20	3765976	3766968	0.000	5	13	7	12	5	12	6	14	9	9	15	4	15	15
CMTM2	16	66613072	66613394	0.000	6	12	5	3	4	10	13	8	10	4	12	4	1	2
ODF3	11	195013	195431	0.000	5	12	8	7	7	7	10	9	9	7	10	6	11	11

Supplementary data 1. Continued

Gene name	Chromosome locus	Gene regio start (GSE42409)	Gene regio end (GSE42409)	p-value (Mann-Whitney U test)	Number of reads													
					OSCC patient 1	OSCC patient 2	OSCC patient 3	OSCC patient 4	OSCC patient 5	OSCC patient 6	OSCC patient 7	OSCC patient 8	OSCC patient 9	OSCC patient 10	OSCC patient 11	OSCC patient 12	Leukocyte pool 1	Leukocyte pool 2
TBX4	17	59532564	59532803	0.000	6	12	3	7	4	8	15	8	7	5	8	5	1	2
AL512362.1	14	104917026	104917422	0.000	7	9	7	10	7	7	11	10	7	7	11	5	15	16
GSC	14	95237544	95237981	0.000	5	5	9	4	7	12	6	8	14	12	11	6	2	3
PATZ1	22	31740363	31741170	0.000	7	7	9	5	7	9	16	10	9	3	9	6	2	3
TBC1D3	17	36282803	36283142	0.000	5	11	4	10	4	13	16	8	5	8	11	8	15	14
AL355075.1	14	20903481	20903844	0.000	7	6	8	7	7	6	11	11	6	6	12	6	13	14
SNED1	2	241936268	241936700	0.001	6	8	3	9	9	6	6	13	5	5	9	7	13	12
AP001476.3	21	47456602	47457078	0.001	4	10	6	9	5	7	7	10	6	10	9	6	14	13
AC068993.1	12	79187669	79188167	0.001	4	7	4	8	5	7	9	10	6	5	11	5	10	10
PCK2	14	24562608	24563016	0.001	4	11	2	10	4	6	9	13	8	6	7	6	13	12
AC007189.1	2	49142926	49143500	0.001	8	12	13	11	7	6	21	15	14	8	18	10	19	18
DIP2C	10	737199	737975	0.001	11	17	10	7	6	4	16	11	8	10	10	5	16	15
C20orf197	20	58629936	58630496	0.001	1	10	4	9	7	13	8	10	10	3	9	13	3	2
5_8S_rRNA	16	33964201	33964553	0.001	8	12	8	9	3	5	6	16	11	5	9	7	13	13
AC021016.2	2	219218778	219219347	0.001	8	9	7	6	5	8	10	11	9	6	13	7	13	14
AC010928.2	18	58329757	58330190	0.001	9	13	6	7	3	4	12	6	12	3	7	7	12	12
FERMT3	11	63974229	63974772	0.001	5	5	5	7	5	9	11	6	8	5	10	7	1	2
CHST6	16	75529780	75530072	0.001	2	15	8	4	5	6	6	13	4	5	15	5	14	13
ATL3	11	63439439	63440134	0.001	5	11	7	8	5	5	9	12	7	7	11	6	11	11
RP11-165M6.1	13	107078138	107078692	0.001	12	6	5	6	7	5	12	11	7	7	13	5	12	12
RPL13AP3	14	56233075	56233429	0.001	7	13	10	7	9	8	9	13	12	6	12	9	15	14
OR1M1	19	9204180	9204744	0.001	6	11	7	7	6	4	11	11	10	7	8	4	12	13
AC131097.1	2	242845825	242846198	0.001	7	12	8	3	6	4	7	12	8	5	11	9	12	13
U3	17	42380910	42381319	0.001	6	10	4	6	7	4	8	6	12	6	7	7	4	4
WFIKKN1	16	678644	679024	0.001	7	12	3	9	8	10	12	13	10	7	11	14	15	14
RPS10L	20	820108	820639	0.001	7	9	5	8	4	9	5	9	10	6	10	6	10	10
AL135798.1	1	117284700	117285259	0.001	7	17	11	16	12	9	14	14	15	9	17	8	17	18
AL356957.13	1	149287471	149287898	0.001	10	5	6	6	5	9	9	11	6	5	10	6	3	2
AC008069.2	2	17036367	17036607	0.001	8	11	7	6	7	9	13	12	6	10	13	4	13	14
AL008723.1	22	32665325	32665766	0.001	6	14	9	8	4	11	7	14	7	8	11	6	13	14
ZNF547	19	57873156	57873573	0.001	4	11	11	4	5	5	8	11	10	8	13	3	12	12
IGHA1	14	106174533	106175030	0.001	8	14	6	13	13	7	10	10	16	11	15	7	15	15
BX322557.4	21	46772281	46773134	0.001	6	12	3	11	6	4	9	18	8	7	8	6	13	14
HES5	1	2463378	2464184	0.001	8	13	5	10	9	10	6	14	6	7	9	11	14	13
AP001466.1	21	15308923	15309360	0.001	5	10	4	5	9	9	10	12	7	6	10	3	11	11
C2orf85	2	242812123	242812826	0.001	7	14	7	6	7	9	10	10	14	10	13	7	13	13
AL451069.4	10	134243532	134244554	0.002	9	11	6	7	8	11	7	12	12	5	14	6	13	14
FCN3	1	27702197	27702709	0.002	6	10	6	5	6	6	9	8	6	4	9	7	9	9
AL139188.2	13	30438274	30438770	0.002	6	17	8	7	3	6	8	18	10	9	15	9	15	16
TMEM132C	12	128899599	128900228	0.002	5	13	5	8	4	12	10	13	12	4	10	9	14	13
AC074212.1	19	46236309	46236955	0.002	7	11	10	12	6	6	16	17	11	11	15	7	20	22

Supplementary data 1. Continued

					Number of reads												
Gene name	**Chromosome locus**	**Gene regio start (GSE42409)**	**Gene regio end (GSE42409)**	**p-value (Mann-Whitney U test)**	**OSCC patient 1**	**OSCC patient 2**	**OSCC patient 3**	**OSCC patient 4**	**OSCC patient 5**	**OSCC patient 6**	**OSCC patient 7**	**OSCC patient 8**	**OSCC patient 9**	**OSCC patient 10**	**OSCC patient 11**	**OSCC patient 12**	**Leukocyte pool 1**	**Leukocyte pool 2**
C21orf77	21	33948372	33948737	0.002	7	11	6	8	5	9	13	9	4	6	10	10	12	13
ACAP3	1	1246319	1246922	0.002	6	18	10	8	15	9	16	17	14	11	15	8	17	17
ADAD2	16	84224448	84224795	0.002	6	11	10	11	5	5	12	16	10	5	15	9	14	14
KIAA0323	14	24897965	24898530	0.002	4	8	5	9	4	6	6	13	7	7	9	7	10	10
C2CD2	21	43374507	43375097	0.002	4	14	10	9	11	7	10	16	16	8	14	3	15	16
TIMM13	19	2428888	2429827	0.002	10	10	12	10	6	4	16	15	9	9	12	8	14	14
AC110299.1	2	242456556	242457232	0.002	4	8	8	8	7	8	8	9	11	5	9	6	12	13
U1	1	146550207	146550837	0.002	7	18	7	4	4	9	22	15	8	5	8	5	2	3
AP005380.2	18	5132955	5133396	0.002	3	11	6	9	7	3	11	12	10	6	13	4	12	13
SETDB1	1	150896594	150896961	0.002	8	6	3	9	6	4	12	10	8	4	8	4	10	10
AC103563.10	2	95638175	95638602	0.002	3	9	8	6	6	4	6	10	6	9	12	6	10	10
CALM2	2	47405104	47405186	0.002	0	1	1	0	1	0	0	1	0	1	1	1	0	0
MSMB	10	51548149	51548150	0.002	1	0	1	0	1	0	1	0	1	1	1	0	0	0
GLS2	12	56881692	56881936	0.002	0	0	1	0	1	1	1	1	1	0	1	0	0	0
HSD11B2	16	67463366	67463367	0.002	0	0	1	1	1	0	1	0	1	0	1	1	0	0
ETV2	19	36132506	36132707	0.002	0	1	0	1	1	0	1	1	0	1	1	0	0	0
FCRLB	1	161689631	161689644	0.002	0	1	1	1	0	0	1	1	0	1	1	0	0	0
LIPA	10	91175701	91176201	0.002	3	10	7	5	4	8	10	10	7	7	10	5	10	10
SYT14	1	210111479	210111996	0.003	9	4	3	3	4	12	16	12	8	5	4	2	2	1
SKI	1	2157600	2158529	0.003	8	8	4	7	7	8	6	13	6	5	9	7	12	11
ELF5	11	34534937	34535481	0.003	7	13	7	6	4	7	7	15	4	7	16	9	13	13
AC133919.5	16	90160195	90160692	0.003	6	14	8	6	8	6	10	11	11	4	7	7	5	5
AC012075.1	2	81694490	81694825	0.003	5	13	6	9	4	8	8	17	8	6	14	3	14	13
SFRS6	20	42084031	42084724	0.003	7	8	3	5	8	7	5	10	6	9	8	6	9	9
AC006269.1	17	53638377	53639063	0.003	6	16	10	6	7	7	17	15	14	8	13	7	15	16
AC018804.7	2	130986044	130986416	0.003	10	9	7	7	6	4	13	16	10	10	12	5	13	14
AC025279.1	16	29300194	29301153	0.003	5	9	6	8	9	9	4	9	12	12	12	7	13	12
hsa-mir-410	14	101532662	101533132	0.003	3	11	5	12	7	7	5	13	13	6	10	5	12	13
WDR24	16	738987	739624	0.003	19	15	12	14	8	8	15	15	18	8	19	10	18	18
LRRC30	18	7231085	7231660	0.003	6	19	5	10	4	6	13	13	12	8	11	4	15	14
C16orf81	16	89225822	89226056	0.003	5	13	5	10	8	9	7	12	9	3	12	8	12	13
C17orf62	17	80409641	80409982	0.003	7	9	7	4	7	6	9	9	6	7	10	4	5	5
AC080112.1	17	38523323	38523956	0.003	6	9	6	8	7	4	5	13	7	7	10	7	10	10
F2	11	46741219	46741868	0.003	9	15	7	8	8	12	7	15	8	7	11	7	14	13
AL359457.2	13	20134785	20135212	0.003	5	12	8	6	5	6	6	16	10	8	11	7	13	12
AP001266.1	11	65546062	65546392	0.003	9	13	10	9	6	8	13	12	16	12	14	9	14	14
AL356961.2	13	112760878	112761112	0.003	8	4	8	4	1	9	17	11	7	1	9	2	2	1
SYT8	11	1846938	1847982	0.004	8	18	12	17	10	14	10	28	6	9	21	11	20	21
GPR25	1	200842396	200842812	0.004	7	7	8	7	9	14	19	10	7	2	11	10	5	4
C2orf65	2	74875016	74875561	0.004	7	9	6	6	8	8	7	12	7	4	12	5	5	5
CASP7	10	115478883	115479141	0.004	6	9	6	7	7	6	15	8	12	8	9	6	4	5

Supplementary data 1. Continued

Gene name	Chromosome locus	Gene regio start (GSE42409)	Gene regio end (GSE42409)	p-value (Mann-Whitney U test)	Number of reads													
					OSCC patient 1	OSCC patient 2	OSCC patient 3	OSCC patient 4	OSCC patient 5	OSCC patient 6	OSCC patient 7	OSCC patient 8	OSCC patient 9	OSCC patient 10	OSCC patient 11	OSCC patient 12	Leukocyte pool 1	Leukocyte pool 2
AL591848.2	1	246954217	246954457	0.004	8	13	6	4	6	7	11	9	8	6	11	4	12	11
hsa-mir-381	14	101512070	101512894	0.004	6	14	5	6	6	7	9	14	13	7	15	9	14	13
PYY2	17	26553901	26554658	0.005	7	10	9	5	9	6	13	14	10	5	11	5	12	13
AP000345.1	22	23909004	23909277	0.005	5	11	8	5	4	7	14	11	8	5	10	6	11	11
CTA-299D3.1	22	48943316	48944061	0.005	3	13	6	10	6	6	12	9	10	9	6	5	11	11
AL122127.9	14	106351596	106351950	0.005	2	8	11	9	5	7	6	10	9	10	8	6	5	5
CHRM4	11	46407278	46407882	0.005	12	16	9	10	11	9	14	11	9	10	13	7	15	14
AP001623.1	21	43720930	43721824	0.005	6	8	8	8	6	7	13	9	7	4	10	5	10	10
BSND	1	55464529	55465160	0.005	6	9	7	5	6	6	7	13	4	7	8	5	11	10
ZNF570	19	37959287	37959750	0.005	7	15	8	6	4	8	11	13	10	4	7	10	12	13
MAFB	20	39319652	39320415	0.005	8	12	11	6	6	7	17	26	5	7	9	4	4	3
C13orf36	13	37247969	37248446	0.005	11	2	6	5	3	10	2	16	3	4	8	5	2	2
PPPDE1	1	244814626	244815003	0.006	10	7	3	6	8	4	10	9	3	7	11	9	10	10
ZNF583	19	56916205	56916724	0.006	7	10	5	9	12	6	11	8	10	7	12	7	11	11
AP001931.1	11	57520088	57520392	0.006	9	10	11	8	7	10	14	15	12	11	7	5	7	7
TUBGCP2	10	135125479	135125859	0.006	4	9	6	9	6	5	6	8	11	5	11	4	11	10
AQP5	12	50355718	50356251	0.006	3	16	6	9	6	6	9	10	11	9	12	6	13	12
CCDC79	16	66835674	66836220	0.006	7	11	10	8	8	6	8	13	9	6	14	8	13	12
DLGAP1	18	3879613	3879881	0.006	6	26	8	11	7	4	14	21	14	8	12	9	18	18
MASP2	1	11108391	11108763	0.006	7	7	3	9	6	6	8	12	5	8	8	7	5	5
SSU_rRNA_5	21	9826641	9826839	0.006	28	10	16	76	35	5	12	37	12	30	49	25	8	9
FAM38A	16	88804843	88805299	0.006	8	13	7	9	6	12	7	18	9	4	9	11	13	13
CCDC79	16	66835180	66835673	0.007	8	18	13	8	8	7	13	14	11	8	13	7	14	15
AL691429.2	10	134778953	134779462	0.007	6	10	7	9	3	7	7	11	13	6	12	4	12	11
C15orf60	15	73735188	73735531	0.007	6	13	5	10	8	4	12	15	4	10	10	6	12	13
CCL15	17	34330963	34330964	0.007	1	1	0	0	0	1	1	0	1	1	0	0	0	0
RAB22A	20	56884284	56884425	0.007	1	0	1	1	0	0	0	0	1	0	1	1	0	0
RP11-529I10.1	10	103329231	103329589	0.007	0	0	1	1	1	0	0	1	1	1	0	0	0	0
HSD17B12	11	43702084	43702130	0.007	1	0	1	0	0	1	1	0	0	1	1	0	0	0
GYLTL1B	11	45944422	45944514	0.007	1	1	1	0	0	0	1	0	1	1	0	0	0	0
AP003108.2	11	61276076	61276077	0.007	0	0	1	1	0	0	0	1	1	1	1	0	0	0
BCAT1	12	25101998	25102064	0.007	0	0	1	1	0	1	0	0	1	1	1	0	0	0
AC084398.1	12	102323488	102323489	0.007	1	1	0	0	1	0	1	0	0	1	0	1	0	0
AB019437.11	14	107150907	107150953	0.007	0	1	0	1	0	0	0	1	1	1	1	0	0	0
ARNT2	15	80696362	80696410	0.007	1	0	0	1	1	0	0	0	1	1	1	0	0	0
PLD6	17	17109465	17109466	0.007	1	0	0	1	1	0	1	0	0	1	0	1	0	0
MFAP4	17	19290689	19290762	0.007	1	0	0	1	0	1	0	1	0	1	1	0	0	0
SECTM1	17	80291235	80291459	0.007	1	0	1	1	0	0	0	1	1	0	1	0	0	0
MYO1F	19	8644031	8644155	0.007	1	1	0	1	0	0	1	0	1	1	0	0	0	0
C1orf113	1	36772133	36772221	0.007	0	0	1	0	0	1	1	1	0	1	1	0	0	0
CACNA1S	1	201082700	201082731	0.007	0	0	1	1	1	0	1	0	0	0	1	1	0	0

Supplementary data 1. Continued

					Number of reads												
Gene name	Chromosome locus	Gene regio start (GSE42409)	Gene regio end (GSE42409)	p-value (Mann-Whitney U test)	OSCC patient 1	OSCC patient 2	OSCC patient 3	OSCC patient 4	OSCC patient 5	OSCC patient 6	OSCC patient 7	OSCC patient 8	OSCC patient 9	OSCC patient 10	OSCC patient 11	OSCC patient 12	Leukocyte pool 1	Leukocyte pool 2
TMEM18	2	678865	678866	0.007	1	0	0	1	1	0	0	1	0	1	1	0	0	0
ZFAND6	15	80350338	80350339	0.007	0	1	1	0	0	1	0	1	0	0	1	1	1	1
U6	1	22314468	22314516	0.007	0	1	0	1	0	0	1	0	1	1	1	0	1	1
SPI1	11	47400930	47401026	0.007	1	0	0	1	0	1	0	0	1	0	1	1	1	1
SLC15A3	11	60720092	60720229	0.007	0	0	0	1	1	0	1	1	0	0	1	1	1	1
AP000770.1	11	116510340	116510341	0.007	1	0	0	1	1	0	0	1	1	0	0	1	1	1
SCARNA11	12	8748456	8748579	0.007	1	1	0	1	0	0	0	0	1	0	1	1	1	1
COMP	19	18903207	18903230	0.007	0	0	1	1	0	0	1	1	0	0	1	1	1	1
INSM1	20	20348794	20348956	0.007	0	0	1	1	1	0	1	1	0	0	1	0	1	1
C16orf81	16	89226057	89226378	0.007	7	12	6	12	8	15	9	11	10	5	15	9	13	14
AC012652.1	15	41521764	41522036	0.007	8	8	2	9	11	5	7	10	5	7	11	7	10	10
CNIH	14	54910018	54910240	0.007	7	13	7	10	3	6	11	13	12	7	15	4	15	17
SPO11	20	55904448	55905206	0.008	6	16	8	9	8	18	7	15	6	11	14	6	18	16
AC018755.9	19	52101660	52102180	0.008	6	15	8	11	11	6	11	22	8	12	14	8	16	15
AC008271.1	2	15830701	15831610	0.008	10	8	7	9	7	13	17	13	11	14	14	9	8	8
TACR2	10	71175640	71176127	0.008	8	8	7	5	6	12	17	12	9	8	8	7	12	13
5S_rRNA	1	228770930	228771604	0.008	14	29	7	6	2	8	32	30	7	16	23	17	31	27
CDKN3	14	54861108	54861777	0.008	8	9	7	5	8	9	10	12	14	6	9	5	11	11
AL356957.13	1	149287899	149288411	0.008	8	9	6	7	4	5	10	9	7	3	15	3	4	4
AC118470.1	1	247802955	247803176	0.009	12	7	5	4	4	7	20	7	6	6	5	2	3	2
AL139161.2	1	236136540	236137128	0.009	6	6	7	5	6	6	9	7	8	7	9	5	8	8
TMEM85	15	34515638	34515959	0.009	3	10	6	7	7	6	7	7	7	9	11	7	10	11
MRPL28	16	422537	423002	0.009	9	10	5	5	9	6	8	11	19	6	10	5	13	12
Y_RNA	14	100048449	100049145	0.009	6	14	6	13	8	5	14	13	15	8	7	8	13	14
AL928742.3	14	106004966	106005349	0.010	5	10	7	7	6	13	4	10	16	9	21	5	14	14
CALML5	10	5540692	5541293	0.010	4	15	7	8	8	4	9	15	4	8	16	11	13	13
INHA	2	220431911	220432323	0.010	8	8	4	12	6	10	11	9	11	5	17	4	13	12
GPS1	17	80008024	80008393	0.010	5	10	7	13	9	7	15	13	14	8	13	6	13	14
AC011491.1	19	6378815	6379216	0.011	9	14	7	6	6	5	13	11	7	6	10	4	12	11
AC104841.1	2	242165415	242165901	0.011	7	12	8	8	8	6	14	13	12	8	8	8	7	7
AL391244.1	1	1354451	1355097	0.011	10	14	11	13	8	12	8	17	14	11	18	6	15	15
ESPNP	1	17046419	17046814	0.011	8	15	10	7	6	7	11	10	8	6	19	10	17	15
RAB1B	11	66033857	66034662	0.011	11	9	4	10	8	8	11	19	13	7	9	7	17	15
TMEM79	1	156251158	156251235	0.012	0	1	1	0	1	0	1	1	0	0	2	0	0	0
GGT5	22	24642529	24642530	0.012	0	1	0	1	1	0	0	2	0	1	1	0	0	0
ALKBH7	19	6369985	6370742	0.012	6	7	7	7	6	6	11	8	7	7	11	4	9	9
BAIAP2	17	79006444	79007013	0.012	11	19	12	6	7	10	17	18	17	8	14	7	16	17
AL136038.2	14	64061697	64062069	0.012	11	22	8	9	10	5	13	17	19	13	16	5	17	17
AL358176.2	1	240799808	240800561	0.012	3	12	5	8	5	9	14	15	7	10	11	5	12	12
TBX4	17	59533693	59534236	0.012	6	11	9	6	5	7	15	11	10	6	13	5	3	1
GZMM	19	543219	543909	0.012	5	10	4	5	6	6	13	7	7	6	8	5	9	9

Supplementary data 1. Continued

Gene name	Chromosome locus	Gene regio start (GSE42409)	Gene regio end (GSE42409)	p-value (Mann-Whitney U test)	Number of reads													
					OSCC patient 1	OSCC patient 2	OSCC patient 3	OSCC patient 4	OSCC patient 5	OSCC patient 6	OSCC patient 7	OSCC patient 8	OSCC patient 9	OSCC patient 10	OSCC patient 11	OSCC patient 12	Leukocyte pool 1	Leukocyte pool 2
CATSPER1	11	65793385	65793796	0.013	8	11	8	10	9	17	14	9	11	10	12	6	14	13
BACH1	21	30670042	30670853	0.013	8	8	4	9	8	5	5	14	10	7	7	8	10	10
P4HA3	11	74022781	74023219	0.013	3	5	6	7	9	8	10	9	9	5	11	4	5	5
MSGN1	2	17997725	17998458	0.014	6	8	5	5	11	9	8	10	10	6	7	6	10	11
BTBD6	14	105713084	105713865	0.014	8	17	10	9	7	7	7	14	16	8	11	6	14	13
AL359737.3	13	19173660	19174402	0.014	5	8	5	10	5	6	10	11	10	7	6	3	5	5
PLEKHN1	1	900206	900744	0.015	8	12	7	6	7	8	11	11	6	10	11	7	12	11
GDF2	10	48416585	48416977	0.015	2	11	10	12	5	5	10	10	7	7	11	3	13	15
RRP15	1	218457065	218457480	0.015	9	6	7	6	7	6	15	10	7	3	9	4	11	10
RASGRF1	15	79382595	79382766	0.015	8	1	0	2	2	9	11	11	1	1	3	2	1	0
AC104024.2	17	16884183	16885006	0.015	3	7	6	7	5	8	9	9	5	8	9	4	10	9
FAM108A6	22	22471677	22472380	0.015	6	10	3	9	5	11	17	6	9	7	13	4	5	5
NKX2-2	20	21496275	21496756	0.016	0	1	2	6	4	10	1	12	0	6	10	2	1	1
GBP5	1	89739002	89739591	0.016	2	13	10	9	6	4	13	15	11	4	8	9	12	12
RASGRP1	15	38857242	38857791	0.016	7	6	3	11	8	8	10	14	13	5	9	7	12	11
ATHL1	11	289630	290144	0.016	6	14	7	15	7	12	5	14	15	9	18	6	7	7
AL451043.2	1	147716091	147716663	0.016	13	21	13	11	13	10	29	16	18	16	25	15	21	22
RP4-697K14.1	20	62199420	62200049	0.016	10	13	11	6	5	12	14	14	9	4	13	7	3	5
RUSC1	1	155290535	155291063	0.016	5	1	6	6	3	12	1	7	12	5	13	3	3	2
AC124861.4	2	241196681	241197295	0.017	7	16	9	7	7	14	12	17	13	8	13	10	14	14
USP18	22	18631319	18631711	0.017	9	14	6	12	7	9	12	10	7	8	9	8	7	6
AC009237.7	2	96190971	96191608	0.017	8	18	11	11	5	9	15	13	13	9	19	7	15	15
FAM3B	21	42675620	42676210	0.017	9	13	6	12	3	4	21	16	6	10	9	3	14	16
C11orf85	11	64739717	64740014	0.017	5	9	9	6	5	8	16	10	6	5	8	4	4	5
MAD2L2	1	11751298	11751523	0.017	0	0	1	1	0	1	1	0	0	1	1	1	1	1
WFIKKN1	16	680974	681202	0.017	1	0	1	0	0	1	1	0	0	1	1	1	1	1
IRX6	16	55357787	55358034	0.017	1	0	1	1	0	0	1	0	1	1	0	1	1	1
FAM71E1	19	50978981	50980006	0.017	6	7	10	9	9	5	13	8	7	9	15	4	12	11
FKBP4	12	2901927	2902637	0.017	8	11	7	8	5	8	7	11	7	7	14	6	6	5
C16orf81	16	89225495	89225821	0.017	2	13	6	9	8	12	5	18	15	2	16	7	16	14
FAM92A2	15	41455529	41456044	0.018	5	10	6	10	3	6	9	10	8	5	8	4	9	9
RPL12L3	20	19804150	19804670	0.018	3	11	8	7	3	14	4	17	4	7	8	7	12	11
AC138969.3	16	16459071	16459683	0.018	0	10	1	15	5	14	5	9	17	6	19	12	14	15
FSCN2	17	79492961	79493634	0.018	5	6	2	9	9	7	7	8	10	8	14	7	10	10
C1orf159	1	1053304	1053617	0.018	4	14	8	7	5	5	6	17	11	8	8	9	13	15
RBP3	10	48389910	48390847	0.018	6	15	7	11	9	12	17	13	11	6	10	6	14	13
SLA2	20	35274515	35274715	0.019	3	3	2	2	0	10	3	14	0	1	8	4	0	1
COX6A2	16	31439306	31439752	0.019	8	5	10	7	8	4	6	10	11	8	11	6	10	11
NACA2	17	59668192	59668741	0.019	6	10	6	10	8	5	14	10	12	8	13	9	7	7
C13orf35	13	113299262	113300283	0.019	15	8	10	8	6	9	15	11	6	6	12	7	12	12
AC015651.1	17	61926521	61927086	0.019	5	12	2	7	6	9	9	14	9	7	14	5	11	12

Supplementary data 1. Continued

					Number of reads													
Gene name	Chromosome locus	Gene regio start (GSE42409)	Gene regio end (GSE42409)	p-value (Mann-Whitney U test)	OSCC patient 1	OSCC patient 2	OSCC patient 3	OSCC patient 4	OSCC patient 5	OSCC patient 6	OSCC patient 7	OSCC patient 8	OSCC patient 9	OSCC patient 10	OSCC patient 11	OSCC patient 12	Leukocyte pool 1	Leukocyte pool 2
JMJD4	1	227921399	227921906	0.019	3	6	6	7	7	14	13	9	12	5	12	6	11	12
GALNT13	2	154728002	154728308	0.020	9	1	3	1	3	4	14	7	0	4	2	2	1	1
RNF17	13	25337805	25338421	0.020	5	9	10	7	4	6	8	16	8	5	14	8	12	11
AC112777.1	12	20704358	20704532	0.020	9	14	9	21	5	10	8	13	13	6	33	5	19	18
C21orf33	21	45551474	45551798	0.020	3	10	4	14	6	5	10	14	8	4	13	6	12	11
GP1BA	17	4836017	4836468	0.021	8	9	3	9	7	5	16	11	6	8	13	5	12	11
AL592464.2	1	2729504	2730299	0.021	8	17	8	11	8	8	20	18	13	9	14	4	16	15
SNX32	11	65601265	65601550	0.021	0	2	0	3	2	14	0	13	3	2	7	0	0	0
KAT2A	17	40274881	40275820	0.021	9	10	11	12	7	9	11	14	7	6	14	6	15	17
NNAT	20	36149825	36150208	0.021	7	12	7	7	3	11	11	20	10	8	8	5	5	6
KIAA0562	1	3774998	3775624	0.021	6	15	3	8	12	8	5	12	5	9	16	10	13	12
AL117692.1	14	50519321	50519571	0.021	6	9	5	5	9	3	7	12	7	5	10	4	10	9
AL109945.1	1	32815223	32815649	0.022	8	7	6	5	11	10	10	10	9	8	8	5	6	5
hsa-mir-380	14	101491469	101492406	0.022	5	15	7	8	9	12	10	15	13	7	16	6	13	14
GRK1	13	114321368	114322096	0.022	6	15	9	8	5	8	10	19	12	8	12	7	13	13
TCL1A	14	96179944	96180575	0.022	10	6	8	5	5	11	9	17	7	8	8	7	13	15
snoU13	17	77685085	77685964	0.022	3	11	4	8	4	10	7	10	9	5	15	6	12	14
AMN	14	103388267	103388745	0.023	5	12	5	5	6	9	3	14	8	7	11	6	10	11
DSCR4	21	39493391	39493628	0.023	6	12	4	8	9	7	12	15	8	9	6	8	11	11
ASPA	17	3375365	3375814	0.023	8	8	6	6	6	8	15	11	7	8	12	4	13	15
NTN5	19	49176094	49176747	0.023	10	8	12	7	12	8	16	17	8	8	16	8	17	15
NAV1	1	201591881	201592262	0.024	10	20	5	11	7	14	18	10	8	4	16	5	6	7
AC004448.7	17	19396521	19397115	0.024	4	7	8	8	6	5	8	5	6	7	10	7	8	8
NEFH	22	29876170	29876886	0.024	9	12	11	8	7	12	21	16	11	5	10	9	8	7
AL158216.1	1	42506718	42507155	0.025	9	10	4	5	6	10	15	8	8	6	8	4	10	11
PAOX	10	135193152	135194112	0.025	3	8	15	18	10	9	10	19	11	6	10	4	14	14
AC068134.5	2	233252919	233253570	0.025	5	13	8	6	9	8	14	14	8	8	13	2	13	15
MBD3	19	1593742	1594594	0.025	7	7	7	10	7	3	16	12	6	5	14	6	11	12
AP002347.1	11	59665177	59665838	0.025	10	14	10	8	5	7	16	10	8	11	9	10	13	12
P4HA3	11	74021586	74022694	0.026	4	5	9	7	7	9	11	8	10	6	15	9	13	15
ACTRT2	1	2936088	2936661	0.026	5	12	5	5	5	11	12	10	8	3	10	6	12	14
EP400NL	12	132567723	132568038	0.026	5	7	7	10	4	3	11	8	5	8	11	5	5	5
SFT2D3	2	128456522	128456875	0.026	7	20	10	6	11	8	8	15	10	7	19	9	14	15
PROKR2	20	5294594	5294876	0.027	5	19	6	9	9	14	13	15	14	7	12	7	14	14
AC018731.1	2	152042525	152042811	0.028	5	11	10	7	4	7	14	6	8	9	8	7	6	6
AC105272.1	1	104112490	104113282	0.028	8	9	5	5	11	10	9	16	8	5	9	10	11	11
AL034420.1	20	50481186	50481798	0.028	6	12	9	6	8	4	12	10	9	9	8	4	13	15
WDR90	16	697261	697875	0.028	9	11	6	8	5	15	4	11	14	8	12	6	15	13
FAM83E	19	49116077	49116544	0.029	4	17	8	7	6	3	7	16	4	8	7	7	11	11
AP001187.6	11	64658499	64658834	0.029	5	16	9	8	7	9	5	15	10	12	8	10	12	12
PROX1	1	214156052	214156507	0.029	5	2	5	6	4	13	20	10	1	3	11	4	3	1

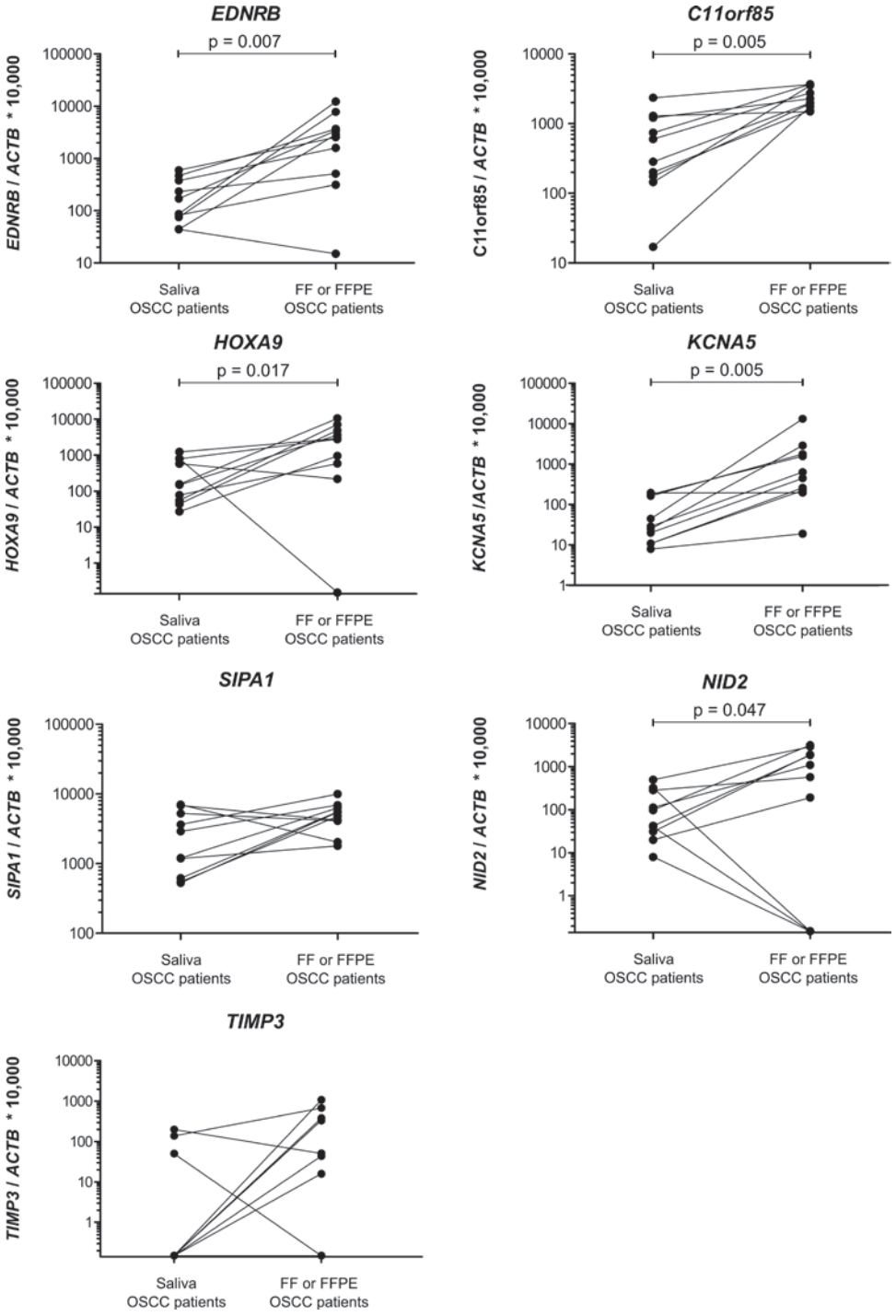
Supplementary data 1. Continued

Gene name	Chromosome locus	Gene regio start (GSE42409)	Gene regio end (GSE42409)	p-value (Mann-Whitney U test)	Number of reads													
					OSCC patient 1	OSCC patient 2	OSCC patient 3	OSCC patient 4	OSCC patient 5	OSCC patient 6	OSCC patient 7	OSCC patient 8	OSCC patient 9	OSCC patient 10	OSCC patient 11	OSCC patient 12	Leukocyte pool 1	Leukocyte pool 2
hsa-mir-663	20	26188963	26189097	0.029	75	40	79	140	38	41	26	34	43	68	78	47	21	2
AL049812.1	20	40626799	40628118	0.030	14	25	14	14	14	9	23	19	13	8	19	9	19	19
MBD1	18	47808654	47809093	0.030	5	16	7	11	5	8	10	9	8	4	18	5	12	12
NPAS4	11	66188392	66189250	0.030	4	5	8	8	3	15	14	11	16	5	15	2	5	3
NTSR2	2	11809606	11810729	0.031	11	13	13	10	8	5	14	13	6	8	16	6	16	14
AL035669.3	20	61406620	61407125	0.032	7	9	4	9	3	8	9	11	5	3	9	8	10	9
AP001476.3	21	47455564	47456395	0.032	2	12	10	17	8	5	9	11	10	8	11	9	15	13
KRT85	12	52760680	52761247	0.032	6	12	5	11	7	9	20	14	10	12	17	4	14	14
5S_rRNA	12	34358079	34358737	0.033	5	12	9	5	5	11	14	17	11	7	12	5	13	15
UTS2R	17	80332010	80332560	0.033	7	15	5	15	10	11	16	15	10	9	17	7	15	17
MYEOV	11	69061709	69062020	0.033	1	10	12	4	5	4	5	6	5	6	27	7	13	12
AC010528.1	16	76268977	76269409	0.033	8	11	7	9	7	8	12	13	9	7	13	6	11	12
PSMA8	18	23713594	23714084	0.034	8	15	7	8	6	6	6	16	20	6	11	7	13	13
TUBB6	18	12306268	12306837	0.034	8	11	7	6	6	2	8	11	3	7	14	4	5	4
CEACAM16	19	45199937	45200643	0.035	9	6	10	8	8	10	6	9	8	8	16	8	6	7
AL357712.1	10	8203710	8204202	0.035	6	10	6	7	8	4	10	9	5	9	9	4	10	9
MRPL20	1	1343891	1344780	0.035	4	15	7	6	8	4	15	10	7	11	10	7	11	12
AC093393.1	2	33952332	33952821	0.035	5	11	10	7	6	10	11	12	12	5	10	6	15	13
AL122018.1	1	236273020	236273412	0.036	6	12	5	9	8	7	11	9	10	5	12	7	15	13
RP11-56M3.1	10	92913356	92913775	0.036	9	13	11	9	6	9	15	7	14	8	12	8	13	12
AC008993.3	19	93193	93664	0.036	8	16	6	7	8	8	13	16	11	12	10	1	6	7
hsa-mir-663	20	26188638	26188962	0.036	76	42	78	139	39	40	24	33	41	66	79	44	21	1
AL391244.1	1	1353425	1353858	0.036	2	8	8	7	5	8	9	10	5	7	11	8	9	9
TM7SF2	11	64878569	64879196	0.037	5	19	5	10	7	11	15	16	13	9	13	9	14	14
AL358237.2	20	58662433	58662514	0.037	0	1	0	6	0	0	6	0	1	0	3	0	3	3
AC215219.3	12	94127	94541	0.038	6	15	5	10	11	4	13	9	15	5	18	4	18	15
C10orf139	10	1205273	1205468	0.038	5	12	8	9	6	3	13	7	6	8	17	6	11	11
MSLNL	16	834001	834714	0.038	3	12	9	11	7	5	5	9	10	9	12	4	11	10
AL355376.2	10	29084569	29085115	0.038	7	10	7	6	6	3	10	8	7	5	12	9	14	12
HTR6	1	19991919	19993142	0.038	2	8	4	12	6	11	12	15	7	5	12	5	11	11
RPS6KB2	11	67194641	67195339	0.038	4	12	9	8	5	2	8	12	4	7	11	7	13	11
KRT71	12	52946425	52946854	0.038	5	16	3	8	10	6	15	6	10	7	16	5	12	12
GPHA2	11	64702199	64702982	0.039	6	13	10	10	7	15	13	21	17	10	15	11	7	9
AC012075.1	2	81694040	81694489	0.040	12	17	12	7	6	8	14	15	13	7	20	9	15	17
CDK2AP1	12	123758033	123758565	0.040	6	9	5	7	5	9	8	11	16	5	9	6	10	11
AC007248.2	2	102866968	102867275	0.040	8	12	5	5	6	10	10	16	8	12	17	5	6	7
AGRN	1	953352	954148	0.041	3	9	8	7	6	9	10	9	12	11	8	8	10	11
ZNRF4	19	5455175	5455830	0.041	7	11	10	13	8	4	19	17	11	8	11	5	14	13
PHACTR4	1	28695091	28695513	0.042	4	9	5	7	9	3	10	12	4	5	14	4	5	4
AC022748.1	15	79042124	79042505	0.042	12	17	5	10	5	9	9	15	15	9	11	12	13	14
CIRBP	19	1268296	1268904	0.042	7	11	6	11	6	6	6	9	5	9	19	10	12	14

Supplementary data 1. Continued

					Number of reads													
Gene name	**Chromosome locus**	**Gene regio start (GSE42409)**	**Gene regio end (GSE42409)**	**p-value (Mann-Whitney U test)**	**OSCC patient 1**	**OSCC patient 2**	**OSCC patient 3**	**OSCC patient 4**	**OSCC patient 5**	**OSCC patient 6**	**OSCC patient 7**	**OSCC patient 8**	**OSCC patient 9**	**OSCC patient 10**	**OSCC patient 11**	**OSCC patient 12**	**Leukocyte pool 1**	**Leukocyte pool 2**
MLNR	13	49794460	49795110	0.042	11	3	12	5	6	6	8	19	7	11	6	5	5	3
FSIP2	2	186603232	186604189	0.043	3	9	7	7	6	5	11	11	9	4	13	4	4	2
FGF3	11	69633336	69634068	0.044	2	2	12	5	1	7	9	6	10	7	38	3	2	2
CLSTN3	12	7280735	7280996	0.044	6	10	9	9	11	7	11	11	11	8	17	5	15	13
DHODH	16	72041197	72041688	0.046	5	8	5	9	6	7	10	6	10	8	11	4	10	9
AC022400.1	10	75491360	75491675	0.046	10	15	10	12	7	5	19	12	10	16	17	6	9	8
KRT33A	17	39506596	39507113	0.046	8	13	6	4	10	8	15	9	8	3	14	8	11	12
AL512638.1	1	115826147	115826583	0.047	6	11	7	6	6	6	8	11	7	5	11	7	9	9
AC092810.2	1	209405064	209405472	0.047	5	16	5	7	7	7	8	15	5	4	13	5	3	5
TNNT3	11	1940716	1941338	0.048	9	10	10	6	5	9	6	12	4	10	12	7	14	12
HSF5	17	56565440	56565821	0.048	11	11	9	9	9	4	10	11	13	6	14	4	6	4
AP002748.2	11	66304685	66305454	0.048	6	11	5	7	4	10	10	5	8	9	16	5	6	5
FAM100A	16	4665443	4666047	0.048	3	11	9	15	5	7	6	9	11	12	13	6	12	11
EDARADD	1	236511487	236512106	0.049	4	13	12	7	6	8	18	16	11	7	14	5	13	13
FAM21C	10	46220649	46221042	0.050	3	5	6	9	5	11	8	9	13	8	10	5	11	13

Abbreviations: Chr, chromosome; TSS, transcription start side; FDR, false discovery rate; bp, base pair).
Location of the methylation as extracted from the "Map of the Human Methylome" [http://www.biobix.be/map-of-the-human-methylome/, BIOBIX (Lab of Bioinformatics and Computational Genomics), Ghent, University of Ghent, Belgium 2014]


EDNRB
p = 0.007
EDNRB / ACTB * 10,000
100000
10000
1000
100
10
Saliva OSCC patients
FF or FFPE OSCC patients
C11orf85
p = 0.005
C11orf85 / ACTB * 10,000
10000
1000
100
10
Saliva OSCC patients
FF or FFPE OSCC patients
HOXA9
p = 0.017
HOXA9 / ACTB * 10,000
100000
10000
1000
100
10
1
Saliva OSCC patients
FF or FFPE OSCC patients
KCNA5
p = 0.005
KCNA5 /ACTB * 10,000
100000
10000
1000
100
10
1
Saliva OSCC patients
FF or FFPE OSCC patients
SIPA1
SIPA1 / ACTB * 10,000
100000
10000
1000
100
Saliva OSCC patients
FF or FFPE OSCC patients
NID2
p = 0.047
NID2 / ACTB * 10,000
10000
1000
100
10
1
Saliva OSCC patients
FF or FFPE OSCC patients
TIMP3
TIMP3 / ACTB * 10,000
10000
1000
100
10
1
Saliva OSCC patients
FF or FFPE OSCC patients

Supplementary data 2. DNA methylation levels of seven OSCC specific markers in saliva and tumor tissues of OSCC patients. Differences in methylation level of the markers between DNA isolated of saliva (saliva patients), fresh frozen (FF tissue) and formalin fixed paraffin embedded tissue (FFPE tissue) of oral squamous cell carcinoma (OSCC) patients. Methylation levels on the x-axis are defined as the average DNA quantity of the gene of interest divided by the average DNA quantity of *ACTB* and then multiplied by 10,000. Saliva and tumor samples from the same patient are connected by a continuous line in the figure. Tumors were defined as methylated if methylation was present in FFPE or FF tumor tissue. Differences between saliva, FF or FFPE were compared using the Wilcoxon rank test, only significant differences ($p < 0.050$) are shown.

Abbreviations: FF, fresh frozen; FFPE, formalin fixed paraffin embedded.

REFERENCES

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015 Mar;65(2):87-108.
2. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin 2013 Jan;63(1):11-30.
3. Van Dijk BA, Gatta G, Capocaccia R, Pierannunzio D, Strojan P, Licitra L, et al. Rare cancers of the head and neck area in Europe. Eur J Cancer 2012 Apr;48(6):783-796.
4. Tabor MP, Brakenhoff RH, Ruijter-Schippers HJ, Kummer JA, Leemans CR, Braakhuis BJ. Genetically altered fields as origin of locally recurrent head and neck cancer: a retrospective study. Clin Cancer Res 2004 Jun 1;10(11):3607-3613.
5. Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. Nat Rev Cancer 2011 Jan;11(1):9-22.
6. Zygogianni AG, Kyrgias G, Karakitsos P, Psyrri A, Kouvaris J, Kelekis N, et al. Oral squamous cell cancer: early detection and the role of alcohol and smoking. Head Neck Oncol 2011 Jan 6;3:2-3284-3-2.
7. Maasland DH, van den Brandt PA, Kremer B, Goldbohm RA, Schouten LJ. Alcohol consumption, cigarette smoking and the risk of subtypes of head-neck cancer: results from the Netherlands Cohort Study. BMC Cancer 2014 Mar 14;14:187-2407-14-187.
8. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011 Mar 4;144(5):646-674.
9. de Bree R, van der Putten L, Brouwer J, Castelijns JA, Hoekstra OS, Leemans CR. Detection of locoregional recurrent head and neck cancer after (chemo) radiotherapy using modern imaging. Oral Oncol 2009 Apr-May;45(4-5):386-393.
10. Arantes LM, de Carvalho AC, Melendez ME, Carvalho AL, Goloni-Bertollo EM. Methylation as a biomarker for head and neck cancer. Oral Oncol 2014 Jun;50(6):587-592.
11. Gasche JA, Goel A. Epigenetic mechanisms in oral carcinogenesis. Future Oncol 2012 Nov;8(11):1407-1425.
12. Sciandrello G, Caradonna F, Mauro M, Barbata G. Arsenic-induced DNA hypomethylation affects chromosomal instability in mammalian cells. Carcinogenesis 2004 Mar;25(3):413-417.
13. Karpf AR, Matsui S. Genetic disruption of cytosine DNA methyltransferase enzymes induces chromosomal instability in human cancer cells. Cancer Res 2005 Oct 1;65(19):8635-8639.
14. Esteller M. Epigenetics in cancer. N Engl J Med 2008 Mar 13;358(11):1148-1159.
15. Lissa D, Robles AI. Methylation analyses in liquid biopsy. Transl Lung Cancer Res 2016 Oct;5(5):492-504.
16. Nunes SP, Moreira-Barbosa C, Salta S, Palma de Sousa S, Pousa I, Oliveira J, et al. Cell-Free DNA Methylation of Selected Genes Allows for Early Detection of the Major Cancers in Women. Cancers (Basel) 2018 Sep 26;10(10):10.3390/cancers10100357.
17. Xu RH, Wei W, Krawczyk M, Wang W, Luo H, Flagg K, et al. Circulating tumour DNA methylation markers for diagnosis and prognosis of hepatocellular carcinoma. Nat Mater 2017 Nov;16(11):1155-1161.

18. Carvalho AL, Henrique R, Jeronimo C, Nayak CS, Reddy AN, Hoque MO, et al. Detection of promoter hypermethylation in salivary rinses as a biomarker for head and neck squamous cell carcinoma surveillance. Clin Cancer Res 2011 Jul 15;17(14):4782-4789.
19. Demokan S, Chang X, Chuang A, Mydlarz WK, Kaur J, Huang P, et al. KIF1A and EDNRB are differentially methylated in primary HNSCC and salivary rinses. Int J Cancer 2010 Nov 15;127(10):2351-2359.
20. Sun W, Zaboli D, Wang H, Liu Y, Arnaoutakis D, Khan T, et al. Detection of TIMP3 promoter hypermethylation in salivary rinse as an independent predictor of local recurrence-free survival in head and neck cancer. Clin Cancer Res 2012 Feb 15;18(4):1082-1091.
21. De Meyer T, Mampaey E, Vlemmix M, Denil S, Trooskens G, Renard JP, et al. Quality evaluation of methyl binding domain based kits for enrichment DNA-methylation sequencing. PLoS One 2013;8(3):e59068.
22. Clausen MJ, Melchers LJ, Mastik MF, Slagter-Menkema L, Groen HJ, Laan BF, et al. RAB25 expression is epigenetically downregulated in oral and oropharyngeal squamous cell carcinoma with lymph node metastasis. Epigenetics 2016 Sep;11(9):653-663.
23. Clausen MJ, Melchers LJ, Mastik MF, Slagter-Menkema L, Groen HJ, van der Laan BF, et al. Identification and validation of WISP1 as an epigenetic regulator of metastasis in oral squamous cell carcinoma. Genes Chromosomes Cancer 2016 Jan;55(1):45-59.
24. Guerrero-Preston R, Soudry E, Acero J, Orera M, Moreno-Lopez L, Macia-Colon G, et al. NID2 and HOXA9 promoter hypermethylation as biomarkers for prevention and early detection in oral cavity squamous cell carcinoma tissues and saliva. Cancer Prev Res (Phila) 2011 Jul;4(7):1061-1072.
25. Mensaert K, Denil S, Trooskens G, Van Criekinge W, Thas O, De Meyer T. Next-generation technologies and data analytical approaches for epigenomics. Environ Mol Mutagen 2014 Apr;55(3):155-170.
26. Brinkman AB, Simmer F, Ma K, Kaan A, Zhu J, Stunnenberg HG. Whole-genome DNA methylation profiling using MethylCap-seq. Methods 2010 Nov;52(3):232-236.
27. BIOBIX (Lab of Bioinformatics and Computational Genomics), Ghent, University of Ghent, Belgium. Available at: http://www.biobix.be/map-of-the-human-methylome/, 2014.
28. Hardcastle TJ, Kelly KA. baySeq: empirical Bayesian methods for identifying differential expression in sequence count data. BMC Bioinformatics 2010 Aug 10;11:422-2105-11-422.
29. Melchers LJ, Clausen MJ, Mastik MF, Slagter-Menkema L, van der Wal JE, Wisman GB, et al. Identification of methylation markers for the prediction of nodal metastasis in oral and oropharyngeal squamous cell carcinoma. Epigenetics 2015;10(9):850-860.
30. van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of

the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 2003 Dec;17(12):2257-2317.
31. Wisman GB, Nijhuis ER, Hoque MO, Reesink-Peters N, Koning AJ, Volders HH, et al. Assessment of gene promoter hypermethylation for detection of cervical neoplasia. Int J Cancer 2006 Oct 15;119(8):1908-1914.
32. Melchers LJ, Clausen MJ, Mastik MF, Slagter-Menkema L, van der Wal JE, Wisman GB, et al. Identification of methylation markers for the prediction of nodal metastasis in oral and oropharyngeal squamous cell carcinoma. Epigenetics 2015;10(9):850-860.
33. Peng M, Chen C, Hulbert A, Brock MV, Yu F. Non-blood circulating tumor DNA detection in cancer. Oncotarget 2017 Aug 4;8(40):69162-69173.
34. Merker JD, Oxnard GR, Compton C, Diehn M, Hurley P, Lazar AJ, et al. Circulating Tumor DNA Analysis in Patients With Cancer: American Society of Clinical Oncology and College of American Pathologists Joint Review. J Clin Oncol 2018 Jun 1;36(16):1631-1641.
35. Boyle JO, Mao L, Brennan JA, Koch WM, Eisele DW, Saunders JR, et al. Gene mutations in saliva as molecular markers for head and neck squamous cell carcinomas. Am J Surg 1994 Nov;168(5):429-432.
36. Jung M, Pfeifer GP. Aging and DNA methylation. BMC Biol 2015 Jan 31;13:7-015-0118-4.
37. Qu C, Sun J, Liu Y, Wang X, Wang L, Han C, et al. Caveolin-1 facilitated KCNA5 expression, promoting breast cancer viability. Oncol Lett 2018 Oct;16(4):4829-4838.
38. Ryland KE, Hawkins AG, Weisenberger DJ, Punj V, Borinstein SC, Laird PW, et al. Promoter Methylation Analysis Reveals That KCNA5 Ion Channel Silencing Supports Ewing Sarcoma Cell Proliferation. Mol Cancer Res 2016 Jan;14(1):26-34.
39. Wada Y, Kubota H, Maeda M, Taniwaki M, Hattori M, Imamura S, et al. Mitogen-inducible SIPA1 is mapped to the conserved syntenic groups of chromosome 19 in mouse and chromosome 11q13.3 centromeric to BCL1 in human. Genomics 1997 Jan 1;39(1):66-73.
40. Minato N, Hattori M. Spa-1 (Sipa1) and Rap signaling in leukemia and cancer metastasis. Cancer Sci 2009 Jan;100(1):17-23.
41. Takahara T, Kasamatsu A, Yamatoji M, Iyoda M, Kasama H, Saito T, et al. SIPA1 promotes invasion and migration in human oral squamous cell carcinoma by ITGB1 and MMP7. Exp Cell Res 2017 Mar 15;352(2):357-363.
42. Wang Y, Chen Y, Chen J, Wang L, Nie L, Long J, et al. The meiotic TERB1-TERB2-MAJIN complex tethers telomeres to the nuclear envelope. Nat Commun 2019 Feb 4;10(1):564-019-08437-1.
43. Shibuya H, Hernandez-Hernandez A, Morimoto A, Negishi L, Hoog C, Watanabe Y. MAJIN Links Telomeric DNA to the Nuclear Membrane by Exchanging Telomere Cap. Cell 2015 Nov 19;163(5):1252-1266.
44. Kuiper R, Broyl A, de Knegt Y, van Vliet MH, van Beers EH, van der Holt B, et al. A gene expression signature for high-risk multiple myeloma. Leukemia 2012 Nov;26(11):2406-2413.