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Loss of heterozygosity of *PTCH1* region in basal cell carcinoma and ovarian carcinoma: comparison of microsatellite analysis and high resolution melting

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1. ABSTRACT

Loss of heterozygosity (LOH) of tumor suppressor genes is a frequent event in tumorigenesis. LOH is most often analyzed by microsatellite typing, but here we offer a fast and efficient method for simultaneous SNP genotyping and mutation scanning, which can also be used for LOH detection. High resoultion melting (HRM) provides simple variant detection, and can be adopted for a wide range of applications. When a melting profile for a specific SNP is determined, the screening can be done without the need for sequencing, and only the melting profiles differing from the established melting profiles should be sequenced or analyzed by other methods. LOH of *PTCH1* gene is often found in a series of different tumors, for example basal cell carcinoma (BCC) and ovarian carcinoma (OC). In this study LOH was detected in 50% of BCC and 27.27% of OC, and the detection rates of microsatellite typing and HRM were comparable. Both methods depended only on the heterozygosity of the loci analyzed, but HRM offers an additional advantage of detection of all sequence variants in the gene of interest.

2. INTRODUCTION

Loss of heterozygosity (LOH) is a change from a state of heterozygosity in a normal genome to a homozygous state in a paired tumor genome. LOH is a sign of somatic deletion and is most often regarded as a mechanism for disabling tumor suppressor genes during the course of oncogenesis (1). Tumor suppressor genes are generally inactivated by an intragenic mutation within one allele and the subsequent loss of the corresponding (wild type) allele (2).

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Identification of deleted regions usually relies on genotyping tumor and counterpart normal DNA with polymorphic DNA markers, such as microsatellites. Microsatellites are short, polymorphic, tandem repeat segments dispersed throughout the human genome. Most procedures to detect LOH are based on comparing alleles in tumor and normal tissue after PCR amplification with fluorescently labeled fragments, followed by capillary electrophoresis on an automated DNA sequencer (3).

High resolution melting (HRM) analysis is a simple, powerful and robust method for detecting DNA sequence variants. The method is based on differences in melting curves caused by variations in nucleotide sequence, detected as the change in fluorescence during the heating (melting) of the PCR product in the presence of the intercalating dye. The advantages of high resolution melting analysis for variant scanning include rapid turn-around times, a closed-system environment that greatly reduces contamination risk and, unlike other methods, no sample processing or separation after PCR. The method is an ideal choice for scanning of novel variants or genotyping known ones. (4).

Patched (*PTCH1*) gene is a tumor suppressor gene associated with development of basocellular carcinomas (BCC) of the skin. It is a member of the Hedgehog-Gli signaling pathway, a pathway that plays a major role in embryonic development and stem cell maintenance. The pathway is activated by binding of Hedgehog (Hh) protein to its transmembrane receptor Patched (Ptch). Ptch is then internalized, and releases its inhibition of Smoothened (Smo) protein, which is transported to the cell surface (reviewed in 5). A phosphorylation cascade is then triggered in the cytoplasm, leading to activation of transcription factor Gli, which translocates to the nucleus and triggers transcription of target genes: *Cyclin D2*, *Cyclin E* (6), members of the Wnt signaling pathway (7, 8), *N-MYC* (9), *PTCH1* (10), *Ceruloplasmin*, *ITIH3* (11), *bcl-2* (12), *FOXM1* (13).

PTCH1 gene is located on chromosome 9, in the chromosome region 9q22.32, with 24 exons streching through 70 kb (14). *PTCH1* gene has one polymorphic CGG repeat marker located directly upstream of the first ATG codon of exon 1b (rs71366293) (15), and at least 48 single nucleotide polymorphisms (SNP) throughout the gene (16), 7 of them common (17).

Germline mutations of the *PTCH1* gene lead to Gorlin syndrome, a syndrome characterized with various developmental malformations and tumors (18, 19, 10). Tumor suppressor role of the gene causing the Gorlin syndrome was known even before it was cloned in 1996 (20). Even then LOH analysis was based on microsatellite markers throughout the whole 9q22.31-9q22.33 region. Sporadic mutations of the *PTCH1* gene are involved in a series of sporadic tumors, including BCC (21, 22), medulloblastomas (23), skin trichoepitheliomas (24), esophageal squamous cell carcinomas (25), squamous cell carcinomas (26), breast cancer (23), as well as in odontogenic keratocysts (27).

According to the recent research, Hh-Gli signaling pathway is involved in one third of all malignant tumors, so it is becoming an interesting target for new cancer therapies (28). The role of Hh-Gli signaling pathway in ovarian carcinoma (OC) has been discovered only recently. Unlike healthy epithelial ovarian tissue, OC shows increased protein expression of the members of the pathway (29). Also, ovarian tumors often show LOH of the *PTCH1* gene (30). Benign tumors of the ovary also show promoter hypermethylation (31), while OC do not (32).

We aim to provide evidence that the high resolution melting analysis is a simple, fast, accurate and inexpensive method to detect LOH, comparable to microsatellite analysis in both sensitivity and specificity.

3. MATERIALS AND METHODS

3.1. Patient samples and DNA extraction

Tumor tissue samples and blood samples from BCC patients were collected at Department of Maxillofacial Surgery, Clinical Hospital Osijek, in Osijek, Croatia. Tumor tissue samples and blood samples from OC patients were collected at Department of Obstetrics and

Gynecology, Zagreb University School of Medicine, in Zagreb, Croatia. All samples were collected with patients' consent according to Declaration of Helsinki, and ethical committees of both institutions approved the study. In total 25 BCC samples from 19 patients and 12 OC samples from 12 patients were collected. DNA was extracted from tissues using the standard phenol-chloroform method, and from blood using the desalting method.

3.2. Microsatellite analysis of LOH

LOH analysis was done using fluorescent labeled forward primers, followed by fragmental analysis detection on ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA).

DNA of 25 BCC and 12 OC samples were typed for CGG repeat in 5'UTR of *PTCH1* gene (rs71366293) using following primers: F: 5' CCCCCGCGCAATGTGGCAATGGAA '3 and R: 5' CGTTACCAGCCGAGGCCATGTT '3 (33). Samples that were uninformative were further typed for three additional STR markers (D9S196, D9S287 and D9S180) (17).

PCR conditions for CGG repeat were: initial denaturation at 95°C for 10 min, followed by 30 cycles of 95°C 30s, 61°C 2 min, 72°C 1 min, and final extension at 72°C 30 min using AmpliTaq Gold polymerase (Applied Biosystems, USA).

PCR conditions for the multiplex PCR with other STR markers: initial denaturation at 95°C for 15 min, followed by 19 cycles of 95°C 30 s, 55°C 90s, 72°C 1min, and final extension at 72°C 30 min using QIAGEN Multiplex PCR kit (QIAGEN, Germany).

3.3. High resolution melting analysis of SNPs

PCR reactions were performed in a 10 µl reaction mixture in Roche LightCycler capillaries using the following components: 50 ng template DNA, 0.2 mM dNTPs (Roche, Mannheim, Germany), 0.4 U FastStart Taq DNA Polymerase (Roche), 1x fluorescent dye LCGreen Plus (Idaho Technology, Salt Lake City, ID, USA), 2 mM MgCl2 (Idaho Technology) and forward and reverse primers (0.5 mM each) for each gene segment. PCR reaction was performed in an adapted RapidCycler2 instrument (Idaho Technology). Coding region of the *PTCH1* gene

was split into 24 PCR products, sized between 168 and 428 bp. PCR conditions were optimized to temperatures between 52^{o}C and 64^{o}C for each segment. After 40 cycles of amplification, PCR products underwent an additional 1 min at 98^{o}C and then 5 min at 40^{o}C to promote heteroduplex formation. Each capillary was then transferred to the High Resolution Melter instrument (HR-1, Idaho Technology) for high resolution melting and curve analysis. Samples were melted at 0.2^{o}C/s ramp rate (34). Melting profiles were analyzed with HR-1 software using fluorescence normalization, temperature shift and conversion to difference and derivative plots. Paired blood and tissue samples were compared, and samples showing a difference in melting profiles were sequenced.

3.4. DNA sequencing

Before sequencing, the chosen PCR products were purified with ExoSAP-IT (USB, Cleveland, OH, USA) and then sequenced in both directions using the Big Dye Terminator 1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequencing reaction was performed on an automatic sequencer ABI PRISM 310 Genetic Analyzer (Applied Biosystems), and sequences were analyzed using BioEdit program.

3.5. Statistical analysis

Kappa statistics κ (35) were used for evaluating inter-rater agreement between two LOH analysis methods and κ values were interpreted according to Landis and Koch (36). Fisher's exact test was used for assessing difference in LOH distribution between BCC and OC samples (two-tailed P value < 0.05 was considered statistically significant). Statistical analyses were performed with MedCalc for Windows, version 11.4.2.0 (MedCalc Software, Mariakerke, Belgium).

4. RESULTS

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4.1. Microsatellite analysis

LOH of the *PTCH1* region was determined in 10 of 25 BCC from 9 patients (40%) (Figure 1, **Table 1-left part**). Twelve BCC samples from 11 patients had no LOH, while 3 samples from 2 patients were uninformative for all 4 markers used (D9S196, rs71366293, D9S287 and D9S180). So the prevalence of LOH in BCC was 45.45% (10/22).

Six patients had two BCCs analyzed. Of those, three had LOH in one tumor but not in the other, one had LOH in both tumors, one had no LOH, and one was uninformative for all markers.

In OC samples, LOH of the *PTCH1* region was determined in 3 of 12 cases (25%) (**Table 1-left part**). Seven OC samples had no LOH, and 2 samples were uninformative for all markers analyzed. The prevalence of LOH in OC was 30% (3/10).

4.2. HRM analysis

Using the HRM analysis and sequencing, LOH was detected in BCC samples with SNPs located in exons 5 (c.735A>G), 12 (c.1665T>C, c.1686C>T), 14 (c.2199A>G) and 23 (c.3944C>T), and introns 1 (c.202-539delC), 5 (c.747-55T>C), 10 (c.1504-51C>G) and 15 (c.2560+9G>C) (Figure 2, Figure 3, **Table 1-right part**).

We considered 1 informative SNP enough evidence for confirmation of LOH, and in all samples analyzed all SNPs corresponded together, that is, when more than one SNP was informative they all either showed LOH or did not. LOH was detected in 10 BCC samples, with 1 up to 4 SNPs being informative.

Eleven samples showed no LOH, with 1 to 4 SNPs being informative. Four samples were homozygotic for all *PTCH1* exons analyzed, so no information about LOH could be gathered. The prevalence of LOH in BCCs detected by HRM was 47.6% (10/21)

In ovarian carcinoma samples LOH was found in exons 12 (c.1686CT) and 23 (c.3944C>T), and introns 1(c.202-539delC), 5 (c.747-55T>C) and 15 (c.2560+9G>C). LOH was found in 3 samples, with 4 or 5 SNPs being informative (**Table 1-right part**).

LOH was not found in 7 samples, with 2 up to 6 SNPs being informative. Two samples were homozygotic for all *PTCH1* exons analyzed, so no information about LOH could be gathered. In ovarian carcinomas, the prevalence of LOH detected by HRM was 30% (3/10)

4.3. Comparison of two methods

The inter-rater agreement between microsatellite and HRM LOH analyses obtained a κ value of 0.821, what can be interpreted as very good agreement (**Table 2-upper half**). When we excluded samples that were uninformative by either method, the κ value was even higher: 0.932 (**Table 2-lower half**). For BCC samples, data from microsatellite and HRM analysis corresponded in nine samples that showed LOH, 11 samples that did not show LOH and three samples that were uninformative. The results did not match in two cases: in one case (BCC18) microsatellite analysis showed LOH, while the HRM analysis was uninformative. In this case the microsatellite analysis showed itself to be more accurate. In the second case (BCC21), the microsatellite analysis showed no LOH, while the HRM analysis showed LOH in 4 SNPs. Here the HRM analysis was more accurate.

For OC samples, data from two analyses corresponded in three samples that showed LOH, 6 samples that showed no LOH, and one uninformative sample. The data did not match in two cases. In one case (OC5) the microsatellite analysis showed no LOH, while the SNPs were uninformative, and in the second (OC10), the microsatellite were uninformative; while the SNPs showed that there was no LOH.

Combining the results from both methods, the prevalence of LOH in BCCs was 50.0% (11/22) while in OC samples was 27.27% (3/11). There was no statistically significant difference between the prevalence in BCCs and OC (P=0.278, Figure 4).

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5. DISCUSSION

High resolution melting analysis has mostly been used for mutation scanning or genotyping (detection of SNPs) in a variety of different systems (reviewed in 37). So far only one paper mentions the possibility of LOH detection using melting curve analysis (38), although a similar concept was established previously using denaturing high-performance liquid chromatography (DHPLC) (39). LOH has previously mostly been detected by microsatellite typing (3). More recently, oligonucleotide SNP arrays have become another tool for LOH detection although it is limited by availability (1). HRM has become widely available in a variety of applications, which has been demonstrated by the number of publications in the recent years (reviewed in 4).

In our research we compared the HRM approach with the classic microsatellite analysis. We determined that LOH can be easily detected using HRM when comparing melting curves from blood and tissue samples, on SNP locations. In these cases there is a visible difference in melting curve profiles between tumor and blood sample from the same patient. Usually the difference is a clear shift of the melting profile from a heterozygous towards wild type or homozygous form, indicating complete or partial loss of one of the alleles. When a melting profile for a specific SNP is determined, the screening can be done without the need for sequencing, and only the melting profiles differing from the established melting profiles should be sequenced or analyzed by other methods.

The detection rate between HRM and microsatellite analysis was comparable, and both were dependant on the heterozygosity of the used loci. The prevalence of LOH was 50% for BCC, and 27.27% for OC. In literature the prevalence of LOH in BCCs is between 35 and 75% (40, 22, 41, 42, 26) and 70% for ovarian tumors, both benign and malignant (30). Our data is comparable with published data for BCC, but is significantly lower for OC. That is probably because of the small number of our OC samples, but we are confident in our results, because of high agreement of both methods used.

When we compared the HRM and microsatellite analysis, our data corresponded in most samples. The data differed in four samples, two BCCs and two OC. In one sample (BCC18), microsatellite analysis showed LOH, while it was uninformative for the SNPs. While we can predict that this means the sample has the LOH in the *PTCH1* gene, since the microsatellite marker (D9S180) is almost 2 Mb away, we cannot say anything for certain. In the second BCC case, where the microsatellite analysis showed no LOH, while the HRM analysis showed LOH, we can be 100% confident that there is a LOH in the *PTCH1* gene. In one OC case, where the microsatellite analysis showed no LOH and the SNPs were uninformative, we cannot say for sure if there really was no LOH (again because of the 2 Mb distance of D9S180). For the second OC case, the microsatellites were uninformative, while the SNPs confirmed that there was no LOH. So, while in two cases the microsatellites were more informative, due to the distance of markers used, it cannot be certain that the *PTCH1* gene itself was lost. In the second two cases, where the SNPs were more informative, the data is more certain, because the SNPs are located in the gene itself.

The only limitation of the HRM method (as well as the microsatellite, or SNP microarray) is the need for SNPs. There are some methods for LOH detection that do not need SNPs, such as Multiplex ligation-dependent probe amplification (MLPA) (43), comparative genomic hybridization (CGH) (44), or whole genome sequencing but they are still not widely used.

For researchers interested in complete genetic analysis of a gene, we recommend HRM as the method of choice, due to its ability to perform scanning for point mutations and LOH analysis in the same reaction. Here we show it with the *PTCH1* gene, but the method could be equally applied to any gene containing common SNPs.

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Abbreviations

LOH: loss of heterozygosity, HRM: high resolution melting, *PTCH1*: Patched1 gene, BCC: basal cell carcinoma, Hh: Hedgehog protein, Ptch: Patched protein, Smo: Smoothened protein, OC: ovarian carcinoma, UTR: untranslated region, STR: short tandem repeat, SNP: single nucleotide polymorphism, PCR: polymerase chain reaction, dNTP: deoxyribonucleotide triphosphate, dHPLC: denaturating high performance liquid chromatography, Mb: megabase, MLPA: multiplex ligation-dependent probe amplification, CGH: comparative genomic hybridization, HET: heterozygous, HO: homozygous, N: normal, UI: uninformative, ND: not done

Key words

loss of heterozygosity, high resolution melting, PTCH1, basal cell carcinoma, ovarian carcinoma

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Running title

LOH in *PTCH1*: microsatellites vs. HRM

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7. FIGURE LEGENDS

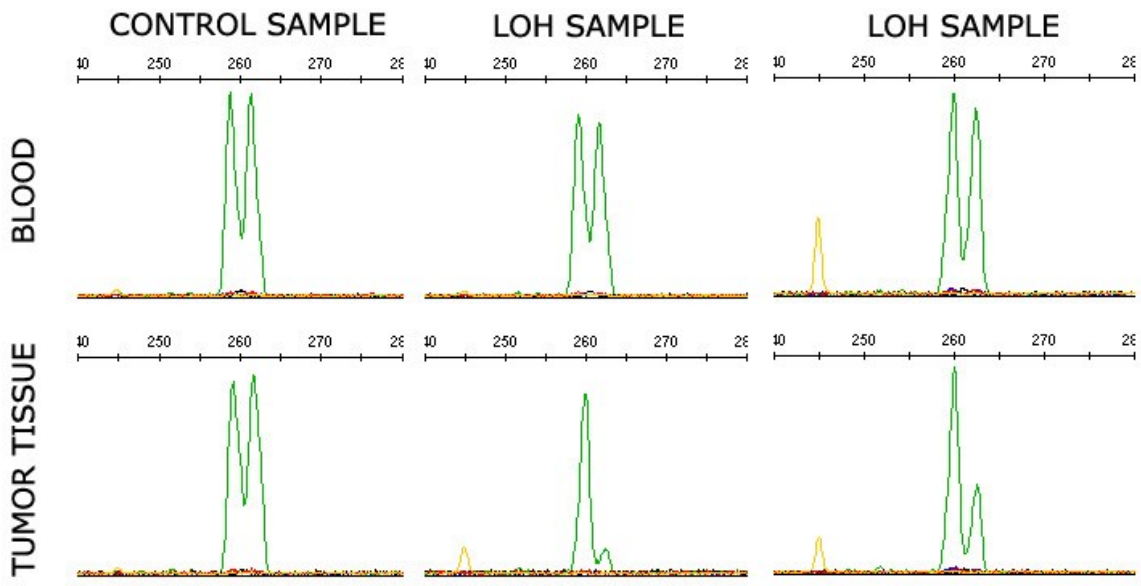

Figure 1. Microsatellite analysis for marker rs71366293 showing two typical LOH results compared to control sample. LOH is calculated from peak height ratio using the following formula: LOH<0.66<normal<1.5<LOH.

Musani V1, Sabol M, Car D, Ozretic P, Oreskovic S, Leovic D, Levanat S. LOH of PTCH1 region in BCC and ovarian carcinoma: microsatellite vs. HRM analysis. Front Biosci (Elite Ed). 2012 Jan 1;4:1049-57. DOI: 10.2741/440 (postprint version)

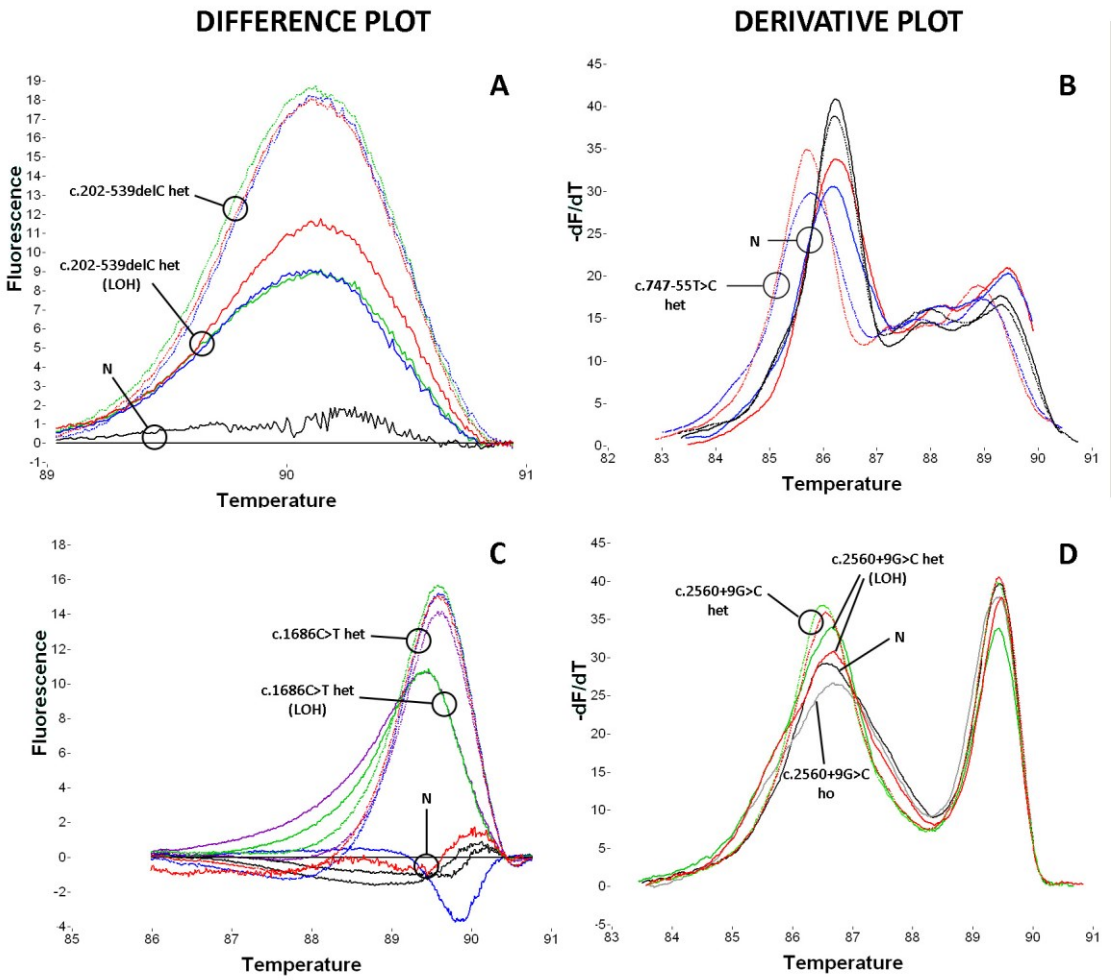

Figure 2. HRM analysis of c.202-539delC (A), c.747-55T>C (B), c.1686C>T (C) and c.2560+9G>C (D) in the *PTCH1* gene. Two different modes of analysis are shown - difference or derivative plots. Normal samples are shown in black, samples homozygous for minor allele in grey, while samples showing LOH are shown in matched color. DNA from blood is shown in dashed lines and DNA from tumor tissue in full lines. LOH samples clearly differ from heterozygous curves. The curves show: partial loss of one allele (A), complete loss of one allele (B) and both partial and complete loss of one allele (C) and (D).

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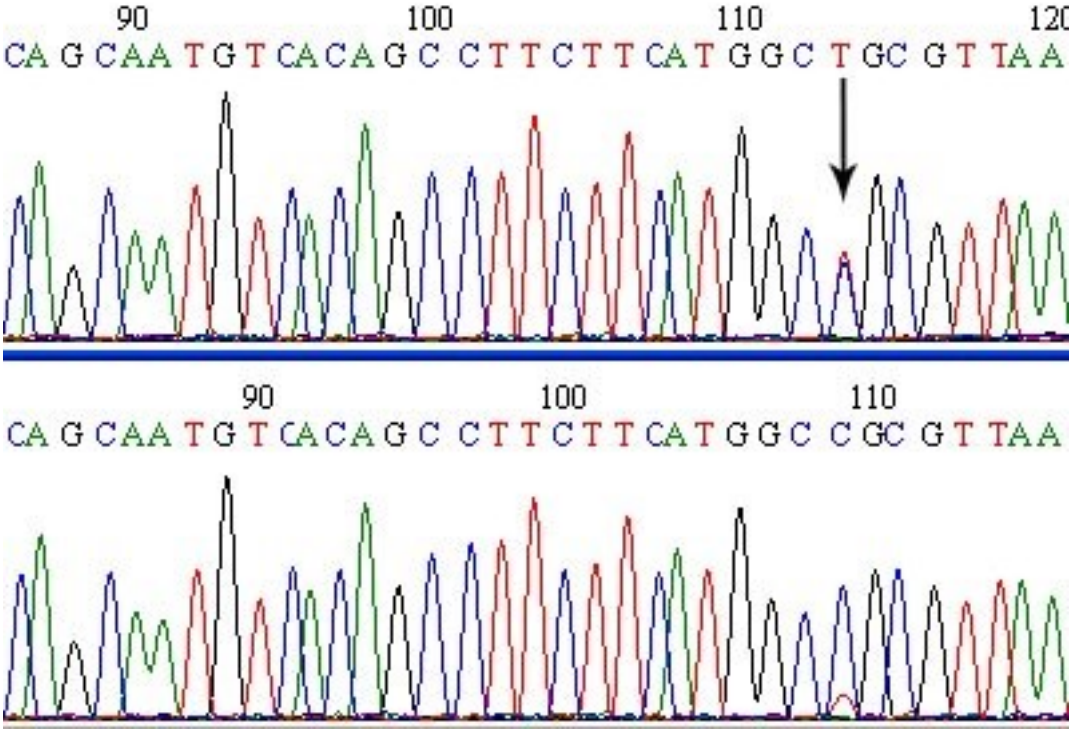

Figure 3. DNA sequencing result showing LOH for c.1686C>T. Upper sequence is heterozygous, while lower shows the loss of one allele (arrow).

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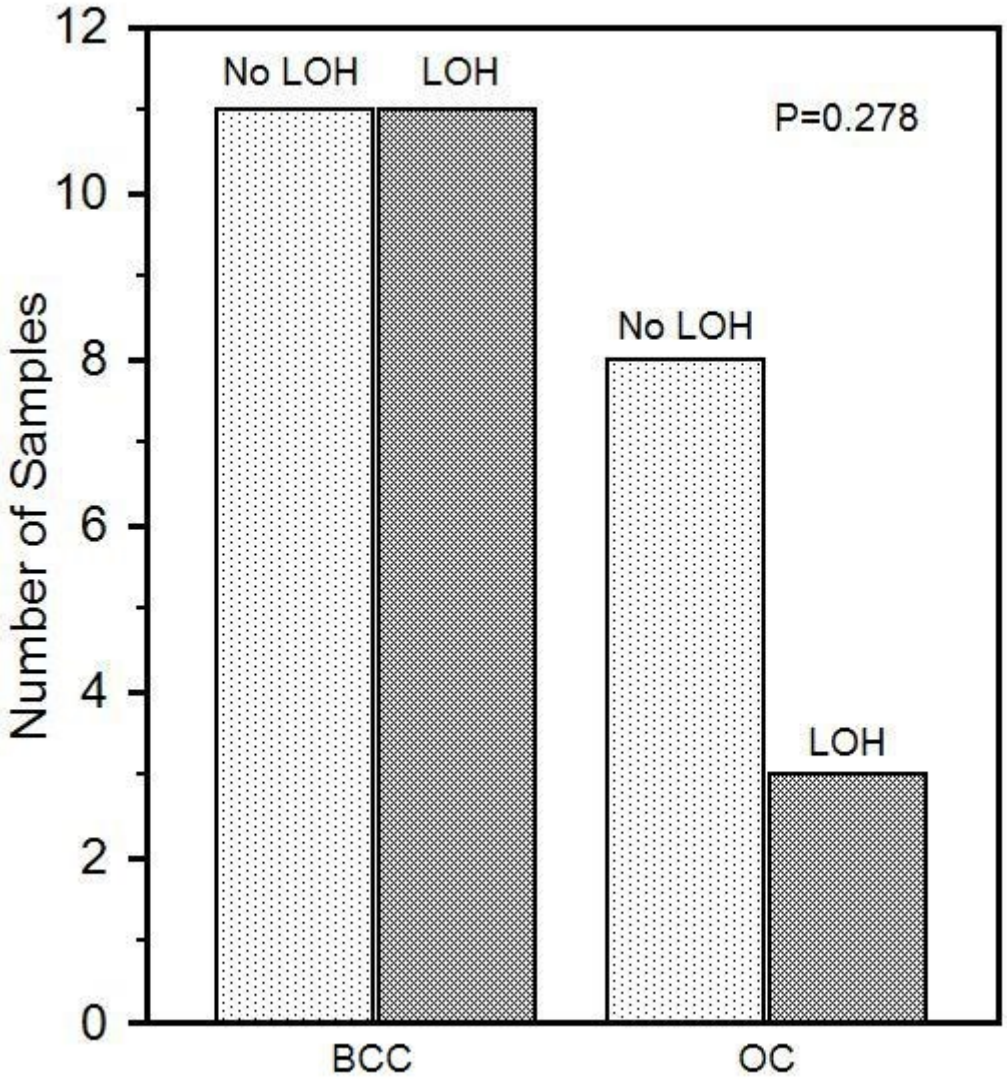

Figure 4. The prevalence of *PTCH1* LOH in BCC and OC. Lighter bars represent samples without LOH, darker bars represent samples with LOH. No statistically significant difference was observed between BCC and OC (P value for Fisher's exact test was 0.278).

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8. TABLES

Musani V1, Sabol M, Car D, Ozretic P, Oreskovic S, Leovic D, Levanat S. LOH of PTCH1 region in BCC and ovarian carcinoma: microsatellite vs. HRM analysis. Front Biosci (Elite Ed). 2012 Jan 1;4:1049-57. DOI: 10.2741/440 (postprint version)

Table 1 – LOH status of BCC (BCC1-BCC25) and OC (OC1-OC12) samples for four microsatellite loci in *PTCH1* gene region and 7 common SNPs detected with HRM in *PTCH1* gene. N – normal, HET – heterozygous, HO – homozygous for minor allele, LOH – loss of heterozygosity, UI – uninformative, ND – not done, - - PCR amplification unsuccessful. No lines between samples – two samples from the same patient.

Marker / Sample	D9S196	rs71366293	D9S287	D9S180	LOH Status	rs11362678 c.202-539delC	rs2297087 c.747-55T>C	rs574688 c.1504-51C>G	rs1805155 c.1665T>C	rs2066836 c.1686C>T	rs2066829 c.2560+9G>C	rs357564 c.3944C>T	LOH Status
BCC1	HET	HO	HO	HET	**no LOH**	N	N	HET	N	N	N	HET	**no LOH**
BCC2	HO	HO	HO	HO	**UI**	N	N	HO	N	N	N	HO	**UI**
BCC3	LOH	HO	HO	LOH	**LOH**	N	HO	N	N	HO	N	LOH	**LOH**
BCC4	HET	HO	HO	HET	**no LOH**	N	HO	N	N	HO	N	HET	**no LOH**
BCC5	HET	HO	HET	HO	**no LOH**	N	HET	N	N	HET	HET	N	**no LOH**
BCC6	LOH	HO	HO	LOH	**LOH**	N	N	LOH	N	N	N	LOH	**LOH**
BCC7	HET	HO	HO	LOH	**LOH**	N	N	LOH	N	N	N	LOH	**LOH**
BCC8	HO	LOH	LOH	LOH	**LOH**	N	N	N	LOH	N	LOH	LOH	**LOH**
BCC9	HO	HO	HO	HET	**no LOH**	N	N	HET	N	N	N	HET	**no LOH**
BCC10	HET	HO	HET	HO	**no LOH**	N	N	HET	N	N	N	N	**no LOH**
BCC11	HET	HO	HET	HO	**no LOH**	N	N	HET	N	N	N	N	**no LOH**
BCC12	HET	HO	HET	HO	**no LOH**	N	N	N	N	N	N	HET	**no LOH**
BCC13	HO	HO	HO	HO	**UI**	N	N	HO	N	N	N	HO	**UI**
BCC14	HO	HO	HO	HO	**UI**	N	N	HO	N	N	N	HO	**UI**
BCC15	LOH	HO	HO	LOH	**LOH**	N	N	LOH	N	N	N	LOH	**LOH**
BCC16	HO	HO	HO	LOH	**LOH**	N	N	LOH	N	N	N	N	**LOH**
BCC17	HO	HO	HO	HET	**no LOH**	N	N	HET	N	N	N	N	**no LOH**
BCC18	HO	HO	HO	LOH	**LOH**	N	N	-	N	N	N	N	**UI**
BCC19	HO	HO	HO	HET	**no LOH**	N	N	HET	HET	N	HET	HET	**no LOH**
BCC20	HO	LOH	LOH	LOH	**LOH**	LOH	LOH	N	N	LOH	LOH	N	**LOH**
BCC21	HO	HO	HO	HET	**no LOH**	N	N	LOH	LOH	N	LOH	LOH	**LOH**
BCC22	HO	HO	HET	HET	**no LOH**	N	N	HET	N	N	HET	N	**no LOH**
BCC23	ND	LOH	ND	ND	**LOH**	N	N	LOH	N	N	LOH	N	**LOH**
BCC24	ND	LOH	ND	ND	**LOH**	N	LOH	LOH	N	LOH	LOH	LOH	**LOH**
BCC25	ND	HET	ND	ND	**no LOH**	N	HET	HET	N	HET	HET	HET	**no LOH**
OC1	-	LOH	LOH	LOH	**LOH**	LOH	LOH	N	N	LOH	LOH	LOH	**LOH**
OC2	HET	HET	HET	HO	**no LOH**	HET	N	HET	N	HET	HET	HET	**no LOH**
OC3	HO	HO	HET	HO	**no LOH**	HO	N	HET	N	HET	HET	N	**no LOH**
OC4	HO	HET	HET	HO	**no LOH**	HET	N	HET	N	HET	HET	HET	**no LOH**
OC5	HO	HO	HO	HET	**no LOH**	N	N	N	N	N	N	N	**UI**
OC6	-	HO	-	-	**UI**	N	HO	N	N	HO	HO	N	**UI**
OC7	HO	LOH	ND	ND	**LOH**	LOH	LOH	HO	N	LOH	LOH	N	**LOH**
OC8	ND	HET	ND	ND	**no LOH**	HET	N	HET	N	N	HET	N	**no LOH**

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OC9	ND	HET	ND	ND	**no LOH**	HET	N	N	HET	HET	HO	N	**no LOH**
OC10	HO	HO	HO	HO	**UI**	N	N	N	N	HET	HET	N	**no LOH**
OC11	ND	LOH	ND	ND	**LOH**	LOH	LOH	N	N	LOH	LOH	N	**LOH**
OC12	ND	HET	ND	ND	**no LOH**	HET	HET	HET	N	HET	HET	HET	**no LOH**

Table 2 – Inter-rater agreement between microsatellite and HRM analyses. Upper half of the table – all samples. Lower half – samples informative for both methods.

		Microsatellite Analysis			
		uninformative	no LOH	LOH	*Total*
High resolution Melting Analysis	uninformative	4	1	1	6 (16.2%)
	no LOH	1	17	0	18 (48.6%)
	LOH	0	1	12	13 (35.1%)
	Total	5 (13.5%)	19 (51.4%)	13 (35.1%)	37
					Kappa 0.821

		Microsatellite Analysis		
		no LOH	LOH	*Total*
High resolution Melting Analysis	no LOH	17	0	17 (56.7%)
	LOH	1	12	13 (43.3%)
	Total	18 (60 %)	12 (40%)	30
				Kappa 0.932