Second primary tumors (SPT) of head and neck. Distinguishing of "true" SPT from micrometastasis by LOH analysis of selected chromosome regions

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The reason of treatment failures in head and neck tumors is often connected with the appearance of second primary tumors (SPT). Three mechanisms of SPT development of clonal or non clonal secondary tumors were described: 1° via micrometastases (clonal); 2° from a common carcinogenic field – Second Field Tumors (SFT – partially clonal); 3° via independent events (from different carcinogenic fields – "true" SPT – not clonal). Assessing the clonality of diagnosed tumors carries important clinical implications including chemoprevention, radiotherapy and general patient management.

In this study a set of 12 microsatellite markers was used to find similarities and/or differences in allelic imbalance patterns between 22 pairs of tumors (the first tumor designate as index and SPT). The aim of the study was to identify a potential clonal origin and progression within given pairs of tumors. The results indicate that within the tumors diagnosed by clinical examination as SPT at least two mechanisms mentioned above should be taken into account as 6/23 (26%) were clonally unrelated ("true" SPT) and 3/23 (13%) carried clonal genetic changes (formation by micrometastasis or SFT). In 14/23 (61%) cases the results were insufficient or ambiguous to determine the clonality status. The final results indicate the complexity of carcinogenesis in these tumors and thus stress that clinical diagnosis of second primary tumors should be considered carefully.

Key words: second primary tumors, head and neck cancer, second field tumors, loss of heterozygosity

Head and neck squamous cell carcinomas (HNSCC) comprises around 5% of all world-wide diagnosed cancer cases and according to the epidemiological data, the incidence of HNSCC in Central and Eastern Europe is still increasing to reach the 4th place as a cause of death among males [24, 14]. Despite advances in surgery and radiotherapy, long-term survival rates of HNSCC have not improved in the recent three decades [13]. The cause of treatment failures is usually connected with the appearance of local relapses, lymph node recurrences, distant metastases and second primary tumors (SPT) [21]. Second primary tumors in patients successfully treated for head and neck cancer are a problem of great clinical concern because statistically up to 36% of patients develop SPT [10, 12]. It is estimated that the second tumors comprise 71% of deaths whereas the first tumor called index only 15%. The overall 5 year survival rate in patients who develop SPT is 8–12% [10, 11]. A high incidence of these events can partially be explained by the "field cancerization" theory, which underlines the dispersed character of precancerous foci in the "condemned mucosa" of the whole upper aerodigestive tract in a typical head and neck cancer patients. Recent studies have proven that in HNSCC patients even in clinically normal mucosa some molecular lesions, typical for neoplastic cells can be found and local recurrence may occur in histopathologically negative surgical margins [20, 26].

Another aspect widely discussed in the literature is a question of clonality of second and index tumors. There are three possible mechanisms of SPT formation of either clonal or non clonal tumors: 1° *via* micrometastases – both tumors clonally related; 2° *via* progression of a carcinogenic field and further accumulation of mutations followed by formation of multiple tumors known as second field tumors (SFT – both tumors clonally related); 3° *via* independent events from different carcinogenic fields ("true" SPT – both tumors clonally unrelated) [1, 26]. Thus, assessing of the clonality status of multiple tumors; tumor and a precancerogenic lesion or tu-

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mor and surrounding filed yields information on the nature of carcinogenesis in a particular patient. The clonality of diagnosed tumors is not only a matter of theoretical dispute but carries important clinical implications. Clonal tumors originating from a carcinogenic field (SFT) should not be treated or treated with great caution with radiotherapy that may cause damage to surrounding cells of the field that already harbors genetic changes. As the field is known to be a potential source of new cancer that is SFT, more radical surgery is recommended to remove a suspicious region entirely [16, 17, 25]. Chemoprevention, for example using isotretinoin, which is currently unlicensed for SPT, or application of retinoids and other anti-oxidants (IFN-α; α-tocopherol) that are thought to prevent oxidative DNA damage and thus further formation of mutations in the field should be advised to the patient [15, 17, 9]. An examination and protection of the entire field to minimize patient's risk of subsequent tumor formation is obvious. On the other hand, there is low risk of carcinogenic side-effect of radiotherapy when normal mucosa surround the tumors. Thus, in order to diminish the recurrence risk (from persistent cancer cells in the surgical area) an intensive post-operational radiotherapy is recommended. Further, occurrence of genetically altered field from which subsequent SFT can arise is a strong argument for patients to quit tobacco and alcohol abuse [15].

An estimation of the ratio of clonal tumors varies drastically among authors and is assessed that the clonality ranges from 23 – 87% [3, 9]. The question remains open and multiple molecular analyses are needed to solve the problem.

In this study LOH analysis was performed to examine the origination and progression of paired laryngeal tumors, clinically classified as index and SPT. We have undertaken a trial to classify and assign the SPT to two different groups according to their clonality status. First group includes tumors carrying clonal genetic changes that are micrometastatic tumors and second field tumors where the expected LOH pattern of the index and second tumor should be identical or at least partially identical. Second group includes the "true" second primary tumors with different LOH patterns for the index and second tumor.

Such classification in our opinion would be benefit for the clinicians in helping patients management and therapy choice. In case of clonal tumors (micrometastases and SFT) we recommend anti-metastatic drugs and chemoprevention for the patients and strict cigarettes and alcohol abstinence. This study is also an attempt to select the most appropriate mikrosatellite markers to create a reliable and not laborious test for the assessment of clonality of head and neck carcinogenic and precarcinogenic lesions.

Material and methods

Archival material stored as paraffin blocks was obtained from the Department of Otolaryngology, K. Marcinkowski Medical University, Poznan, Poland. Genetic imbalances were analyzed in the group of 23 cases (case 9 was divided into two subgroups 9A<-->9B and 9AB<-->9C) (mean age at the first treatment: 59.4 years), and for each of them the DNA was isolated from two (in cases 9 and 21 from three) tumor tissues and blood samples. Material consisted mostly of squamous cell carcinoma (31/46) and basal cell carcinoma (6/46). The clinical data are collected in Table 1. Fourteen patients had identical histology of both analyzed tumor tissues. In eight out of 23 pairs of samples both tumors (multiple primary tumors) were excised simultaneously. Only in four patients both tumors were localized within the same organ (cases 1, 15, 28 and 30). The location of the SPTs was as shown in Table 1 but all remained in the head and neck region (except case 21A). For each paraffin-embedded tumor 10-μm sections were obtained and analyzed by pathologist. From corresponding region of the paraffin block tumor cells were dissected under the microscope. In all cases tumor tissue was deparaffinized by xylene washing, then hydrated and homogenized. DNA was isolated from all samples according to the standard procedures (proteinase K digestion, phenol/chloroform extraction and ethanol precipitation). DNA was PCR-amplified with the use of appropriate primers specific for chosen microsatellite markers (dinucleotide repeats). One primer from each pair was fluorescently end-labeled with Cy5 (Cy5 - Cy5-bis-OSU, N,N'-biscarboxypentyl-5,5'-disulfonatoindodicarbocyanine). PCR products were separated and detected by gel electrophoresis in denaturing 6% polyacrylamide-7M urea using ALF EXPRESS2 sequencer (Pharmacia-Biotech).

In this study a set of 12 microsatellite markers obtained from oligo.pl (Serwis Sekwencjonowania i Syntezy DNA IBB PAN, Poland) was used: D3S1284 (*DUTT1*, 3p12), D3S1300 (*FHIT*, 3p14.2), D3S1317 (*VHL*, 3p25.3), D8S261 (8p21.3), D8S264 (8p23.2), D9S171 (*P16*, 9p21), D9S1870 (*P16*, 9p21), D13S153 (*RB1*, 13q14), D14S1044 (*CHES1*, 14q32), D18S467 (*MADH2,* 18q21.1), D18S474 (*DPC4*, 18q21.1), D18S484 (*DCC,* 18q21.3). Primer sequences of all these markers were obtained from the Genome Database (http://gdbwww.gdb.org).

LOH was calculated from the measured peak heights (relative fluorescence units) by dividing the allele ratio in constitutional DNA by the allele ratio in studied tissue DNA:
$1.5 \leq [n_2/n_1]/[t_2/t_1] 0.5$.

Loss of heterozygosity was scored if a 50% reduction in the peak height of the clinical tissue compared to the peak height of corresponding blood tissue was observed.

Results

In the studied group of tumors allelic imbalances (loss of heterozygosity and microsatellite instability) in at least one locus were found in the majority of studied samples (mean 2.5). Only informative cases (heterozygous at examined locus) were analyzed further. The percentage of informative cases ranged between 17 and 75% of all successfully ampli-

Table 1. Clinical and histopathological characteristic of patients

Case	Primary tumor				Second primary tumor			
	Age (years)	Localization	Histology	TNM	Localization	Histology	TNM	Time of treatment after PT (months)
1	60	larynx	SCC	T2N0	larynx	SCC	T4N1	108
3	73	mandible	SCC	T2N0	larynx	SCC	T2N0	36
6	35	tongue	SCC	T2N0	larynx	SCC	T3N0	0
10	69	auricle L	SCC	T4N0	parotid R	cystadeno-lymphoma	nd	5
11	60	larynx	SCC	T1N0	auricle L	BCC	T2N0	42
12	53	larynx	SCC	T2N0	lung	SCC	nd	0
13	47	larynx	SCC	T3N1	tongue	SCC	T1N0	22
14	48	larynx	SCC	T4N0	alveolar process	SCC	T3N0	0
15	75	larynx	SCC	T2N0	larynx	SCC	T4N0	53
17	73	nasal fossa	SCC	T3N0	parotid L	adenoma polymorph.	nd	0
20	53	larynx	SCC	T3N2	tonsil R	SCC	T1N0	0
21	57	lung	SCC	nd	tonsil L	SCC	T2N1	33
Third primary tumor					uvula	SCC	nd	33
23	77	larynx	SCC	T3N0	parotid R	cystadeno-lymphoma	T2N0	0
28	57	larynx R	SCC	T1N0	larynx L	SCC	T3N0	84
30	56	larynx	SCC	T2N0	larynx	SCC	T3N0	52
58	46	hypophysis	adenoma	nd	larynx	SCC	T3N0	192
80	64	nose vestibule	SCC	T2N0	skin of face	BCC	T1N0	10
92	68	larynx	SCC	T3N1	underlip	SCC	T1	18
9	47	parotid R	cystadeno-lymphoma	T2N0	supra-clavicular	histiocytoma	T4	0
Third primary tumor					underlip	adenoma polymorph.	nd	0
24	34	meninges	meningeoma	nd	supra-clavicular	histiocytoma	nd	14
94	80	auricle L	BCC	T1	nose	BCC	T1	0
100	74	nose	BCC	T1	forehead skin	BCC	T1	16

nd – no data; PT – primary tumor; SCC – squamous cell carcinoma, BCC – basal cell carcinoma

fied markers. The results are summarized in Table 2. LOH in the analyzed group of tumors was found in all chosen markers (mean 37.7%) and ranged between 12 and 73% of informative cases. In contrast, microsatellite instability was observed less frequently (mean 6.6%) reaching about 40% only for marker D3S1300. The frequency of LOH and MSI in analyzed samples is summarized in Table 3.

No differences in the number of observed allelic imbalances were found when tumors were categorized according to TNM system. However, allelic imbalances were more frequent in the large size (T4) tumors (on average 3.8 imbalances per sample) as compared with the other (T1–T3) subgroups (2.3 imbalances per sample).

The aim of this study was to find similarities and/or differences in allelic imbalance patterns between pairs of tumors (index and SPT). To recognize clonality of the analyzed samples the time factor and localization of each particular tumor was considered.

The results of this study have shown unrelated primary tumors in 6/23 (26%) cases and in 3/23 (13%) cases the paired tumors carried clonal genetic changes. In 14/23 (61%) cases received results were insufficient or ambiguous in order to state the clonality status. Within samples representing unrelated primary tumors we singled out these showing different allelic imbalances. These tumors were synchronous with the same histology but developed in different localizations (cases: 6, 14). Interestingly, both of them (6, 14) represented relatively young patients with laryngeal squamous cell carcinoma.

Patients 92 and 100 developed metachronous tumors in different localization with identical histology. In the cases 10 and 17, tumors dissected from different locations were metachronous and had different histology. In the group of patients analyzed in this study two developed three primary tumors. In case 21, all tumors were squamous cell carcinomas, first of the lung and two, which were synchronous, were localized in tonsil and uvula. Our study showed that most likely all these tumors developed independently, however two of them (tonsil and uvula) was localized close to each other, but still carried different allelic imbalances. In case 9, tumors represented different histology but were synchronous. In samples 9A and B the obtained LOH pattern suggest a clonal development. 9C, on the other hand, differs from the other two tumors.

Another group of samples was that with clonal genetic changes found within analyzed loci. Case 1, most probably represent a recurrence – both tumors were localized in the larynx and were metachronous. We found that majority of

Table 2. Summarized results of microsatellite analysis and proposed genetic clonality of analyzed tumors

Case	D3S1284	D3S1300	D3S1317	D8S261	D8S264	D9S171	D9S1870	D13S153	D14S1044	D18S467	D18S474	D18S484	Clonality
1A	no	NI	NI	NI	LOH	LOHrev	NI	NI	NI	NI	LOH	LOH	
1B	LOH	NI	NI	NI	LOH	LOHrev	NI	NI	NI	NI	LOH	LOH	Clonal
3A	LOH	NI	NI	LOH	LOH	NI	NI	NI	LOH	NI	LOH	NI	
3B	no	NI	NI	RET	LOH	NI	NI	NI	RET	NI	RET	NI	Non clonal
6A	LOH	MSI	NI	NI	RET	no	NI	no	NI	LOHrev	LOHrev	NI	
6B	MSI	MSI	NI	NI	LOH	no	NI	no	NI	LOHrev	LOHrev	NI	Non clonal
10A	NI	LOH	NI	NI	LOH	LOH	LOH	LOH	RET	LOH	LOH	NI	
10B	NI	RET	NI	NI	RET	RET	RET	RET	RET	RET	RET	NI	Non clonal
11A	RET	NI	NI	NI	LOH	NI	NI	no	no	NI	RET	NI	
11B	RET	NI	NI	NI	RET	NI	NI	no	LOH	NI	LOH	NI	?
12A	RET	NI	NI	NI	RET	no	RET	NI	RET	NI	LOH	NI	
12B	LOH	NI	NI	NI	LOH	no	LOH	NI	no	NI	RET	NI	Clonal
13A	LOH	LOH	NI	RET	RET	RET	NI	RET	RET	NI	LOH	LOH	
13B	RET	MSI	NI	RET	RET	MSI	NI	LOH	RET	NI	RET	LOH	?
14A	RET	LOH	NI	no	NI	RET	RET	NI	LOH	RET	NI	RET	
14B	LOH	MSI	NI	no	NI	RET	LOH	NI	RET	LOH	NI	LOH	Non clonal
15A	RET	NI	NI	NI	NI	RET	RET	NI	LOH	RET	NI	NI	
15B	no	NI	NI	NI	NI	LOH	RET	NI	RET	LOH	NI	NI	?
17A	NI	LOH	LOH	NI	NI	NI	LOH	NI	NI	NI	NI	LOH	
17B	NI	RET	RET	NI	NI	NI	RET	NI	NI	NI	NI	RET	Non clonal
20A	LOH	RET	NI	RET	RET	LOH	NI	NI	RET	NI	RET	NI	
20B	RET	LOH	NI	MSI	LOH	LOH	NI	NI	LOH	NI	LOH	NI	?
21A	RET	RET	NI	RET	RET	NI	NI	no	NI	MSI	RET	NI	
21B	LOH	RET	NI	LOH	LOH	NI	NI	LOH	NI	RET	RET	NI	
21C	RET	MSI	NI	RET	RET	NI	NI	RET	NI	RET	RET	NI	?
23A	LOH	no	LOH	RET	RET	NI	no	NI	NI	NI	NI	RET	
23B	no	no	LOH	no	RET	NI	no	NI	NI	NI	NI	LOH	?
28A	no	no	NI	NI	RET	MSI	no	NI	NI	LOH	RET	NI	
28B	RET	no	NI	NI	RET	LOH	LOH	NI	NI	RET	RET	NI	?
30A	NI	MSI	RET	no	NI	NI	NI	no	RET	no	NI	LOH	
30B	NI	no	LOH	no	NI	NI	NI	no	RET	no	NI	no	?
58A	LOH	NI	NI	RET	LOH	NI	RET	RET	NI	RET	RET	NI	
58B	RET	NI	NI	no	LOH	NI	LOH	RET	NI	RET	RET	NI	?
80A	NI	RET	NI	RET	RET	RET	NI	NI	NI	NI	NI	no	
80B	NI	MSI	NI	no	RET	RET	NI	NI	NI	NI	NI	no	?
92A	NI	MSI	NI	no	NI	MSI	NI	NI	NI	MSI	LOH	no	
92B	NI	MSI	NI	RET	NI	LOH	NI	NI	NI	LOH	LOH	no	?
9A	RET	RET	NI	NI	RET	LOH	RET	NI	RET	NI	RET	NI	
9B	RET	LOH	NI	NI	RET	LOH	RET	NI	LOH	NI	RET	NI	A→B clonal
9C	MSI	MSI	NI	NI	LOH	LOHrev	RET	NI	MSI	NI	RET	NI	Non clonal
24A	RET	MSI	NI	RET	NI	RET	no	no	NI	NI	NI	NI	
24B	RET	RET	NI	RET	NI	RET	no	RET	NI	NI	NI	NI	?
94A	RET	LOH	NI	RET	NI	NI	RET	NI	NI	RET	NI	no	
94B	RET	RET	NI	RET	NI	NI	RET	NI	NI	RET	NI	no	?
100A	RET	no	RET	no	NI	RET	LOH	RET	NI	NI	RET	NI	
100B	RET	no	RET	no	NI	RET	RET	no	NI	NI	RET	NI	?

NI – non informative, RET – retention of heterozygosity, LOH – loss of heterozygosity, LOHrev – loss of heterozygosity for different alleles, MSI – microsatellite instability, no – lack of PCR product

Table 3. The frequency of LOH and MSI in analyzed samples

	D3S1284	D3S1300	D3S1317	D8S261	D8S264	D9S171	D9S1870	D13S153	D14S1044	D18S467	D18S474	D18S484
PCR amplified markers	41	40	46	36	46	42	41	37	44	44	46	39
Informative cases (%)	31 (75.6)	28 (70)	8 (17.4)	17 (47.2)	30 (65.2)	25 (59.5)	20 (48.8)	10 (27)	19 (43.2)	19 (43.2)	30 (43.2)	11 (28.2)
LOH (% of informative)	10 (32.3)	7 (25)	4 (50)	2 (11.8)	13 (43.3)	11 (44)	7 (35)	3 (30)	6 (31.6)	7 (36.8)	12 (40)	8 (72.7)
MSI (% of informative)	2 (6.40)	11 (39.3)	0	1 (5.9)	0	3 (12)	0	0	1 (5.3)	2 (10.5)	0	0

allelic imbalances were the same, however some discrepancies were also found, for example LOH for marker D9S171 in the case 1, where LOH concerned different alleles. Case 12, represents most likely a micrometastase and case 9AB a SFT.

The mean time of developing a SPT in all cases was 32 months. Interestingly, our results show that clonally related SPT develop much later (mean 36 months) compared to non clonal SPT (mean 6.8 months).

In fourteen samples we could not reveal the clonality of analyzed tumors. In majority of these cases we found non informative, homozygous alleles for microsatellite markers described as early genetic changes in HNSCC. In part of our material we could not successfully receive PCR products, in spite of multiple reactions, most probably due to poor quality of the paraffin embedded archival samples. Another problem appeared in case 24, where most probably the chosen microsatellite markers did not cover the regions changed in meningeomas nor histiocytomas (see also case 9A representing histiocytoma).

Discussion

The phenomenon of multiple malignancies occurring in the upper aerodigestive tract was already explained by a concept of field carcinogenesis. Multifocal carcinomas develop as a result of independent mutations caused by long-term exposure to carcinogens of large areas of the mucosa [23]. An alternative view of the field concept concerns consequences of carcinogen-induced transformation of single cell followed by clonal expansion and by gradual replacement of normal mucosa giving rise to one, large premalignant field [5]. Both theories meet in point, that the risk of head and neck cancer is determined by a balance between exposure to exogenous factors and endogenous host influence understood as genetically-based susceptibility to carcinogen-induced damage [21].

In this study we investigated allelic imbalances using 12 microsatellite markers chosen for the specific locations at chromosomes 3p, 8p, 9p, 13q, 14q and 18q. These markers were selected because the LOH of them was frequently demonstrated in head and neck tumors and they were suggested to be adequate to assess a clonality status [4, 27]. Analyzing pairs of tumors the strongest indication for unrelated origin of particular tumors was when both tumors carried different allelic imbalances both for early and late genetic changes. In this study early changes were investigated by five markers localized on chromosome arms 3p and 9p. Microsatellite markers located at chromosome arms 3p, 9p and 17p are suitable as clonal markers in HNSCC, because the LOH patterns of these markers are relatively stable during tumor progression [27, 15]. In contrast microsatellite markers at chromosomes 8p, 13q, 14q and 18q were recognized as late markers of genetic changes. In HNSCC imbalances concerning microsatellite markers located at 13q, 8p and 18q are associated with tumor progression and poor prognosis [8, 18].

In the examined set it was possible to distinguish two groups of patients in relation to genetic clonality of both tumors. In 3/23 (13%) cases clonality of two tumors could be confirmed. In 6/23 (26%) cases our results indicated a non-clonal origin. The remaining 14/23 (61%) cases could not be ascribed to any of the groups due to ambiguous results, high number of non-informative markers and sometimes no PCR amplification. The results showed that non-clonal origin in SPT (true SPT) was twice as frequent as clonal development. The latter result should be analyzed further including *p53* gene sequencing showing mutation in the majority of HNSCC [2, 25, 27, 28, 15]. The mutation pattern in *p53* gene shows a high variability that can be followed by polyclonal character of transformation [15].

The case 1 is most probably a recurrence. Both tumors share almost the same LOH pattern and the tumor developed in the same anatomical region although the time of SPT formation is extremely long and not typical for relapse.

Case 12, could be an early micrometastases. The "downstream" direction of the formation of the second tumor suggests metastasis of a neoplastic cell with saliva (mucosal erosion). This mechanism was already described for multifocal tumor growth seen in certain types of lung cancer [7].

The case 9A and B represent most probably an expanding carcinogenic field from which two tumors arose. Such field is formed from clonally spread cells but accumulation of further changes is necessary for tumor formation, thus two tumors inherit some common changes from the field but differ in the successive acquired during progression. This is supported by the similar pattern of LOH in markers thought to be an early event in head and neck cancer (D9S171; D3S1284).

The observed changes in the case 13 suggest an expanding field but the anatomical localization of the tumors (larynx,

tongue) would require a field to expand into a large area. Although previous investigations by PARTRIDGE et al [16] and SCHOLES et al [22] showed such possibility, late alterations like LOH in chromosomes 8 and 18 suggest rather a metastasis. *P53* analysis that was proved to be very useful in distinguishing between second lung carcinoma and lung metastases would be most probably the way to solve the problem thus we decide not to assign this case to any of the groups.

The frequency of allelic imbalances in case of the 12 markers was similar beside the high frequency of LOH on chromosome 18 that is associated with poor prognosis [6, 19].

We observed that clonal SPT develop much later than non-clonal tumors. Clonal development implies the presence of cells already harboring early mutations associated with tumor formation thus it seems logically that these cells should enter carcinogenesis earlier than cells without mutations in genes important for tumor formation. Other yet unknown mechanism must cause the observed phenomenon.

Remaining pairs of tumors which most probably were not genetically related represent different allelic imbalances in the tumors and/or LOH was found in opposite alleles, indicating unrelated changes acquired independently.

The clinical definition of multiple or second primary tumor was established in the last century and the criteria for diagnosing independent malignant tumors are well known. A molecular approach, on the other hand, gives a new insight into this phenomenon and shows that second primary tumors recognized according to clinical definition are a divergent group which includes: clonally homogeneous tumors sharing genetic changes entirely or only partially (for example only early changes) and tumors that are absolutely unrelated [26].

Our results confirm and stress the complexity of SPTs formation in the head and neck region. We present alternative ways of tumor formation already in a relatively small group of patients. However, we demonstrated that a small number of 12 microsatellite markers can allow only in some cases to distinguish SPT developed clonally from that of non clonal origin. The relatively high number of unclassified cases 14/23 (61%) indicates that the usage of 12 mikrosatellite markers and stab gel electrophoresis is not sufficient to distinguish the clonality status in most cases. We suggest therefore combination of LOH pattern analysis with *p53* gene mutation analysis in further studies. Such combination should allow the determining of clonality status. This, we hope, would aid clinicians in deciding about chemoprevention, radiotherapy and general patient management.

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