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Droplet digital polymerase chain reaction for detection and quantification of cell-free DNA TP53 target somatic mutations in oral cancer

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Abstract.
BACKGROUND: TP53 mutation is a driver mutation of oral carcinogenesis. This study investigated cancerous and cell-free DNA (cfDNA) in patients with oral squamous cell carcinoma (OSCC) to detect the target hotspot somatic mutation of TP53.
OBJECTIVE: TP53 target hotspot mutations were determined in surgically resected primary tumor samples from 107 OSCC patients.
METHODS: Cancerous and cfDNA samples were examined for mutations through droplet digital polymerase chain reaction (ddPCR) by using mutation-specific assays. The ddPCR results were evaluated alongside clinicopathological data.
RESULTS: In total, 23 cases had target TP53 mutations in varying degrees. We found that OSCC had relatively low cfDNA shedding, and mutations were at low allele frequencies. Of these 23 cases, 13 had target TP53 mutations in their corresponding cfDNA. Target somatic mutations in cancerous DNA and cfDNA are related to cervical lymph node metastasis. The cfDNA concentration is related to primary tumor size, lymph node metastasis, and OSCC stage.
CONCLUSIONS: Our results show that the detection of TP53 target somatic mutations in OSCC patients by using ddPCR is technically feasible. Low levels of cfDNA may produce different results between cancerous tissue and cfDNA analyses. Future research on cfDNA may quantify diagnostic biomarkers in the surveillance of OSCC patients.

Keywords: cfDNA, ddPCR, oral cancer, tp53, somatic mutation

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) includes oral squamous cell carcinoma (OSCC) and is the sixth most prevalent malignancy worldwide [1]. Despite in the past 40 years ample knowledge has been gained regarding the carcinogenesis in HNSCC and OSCC, and a lot of innovative developments have been made in surgery, chemotherapy, and radiotherapy, the prognosis for many HNSCC types has not considerably improved [2,3]. The Taiwan Ministry of Health and Welfare (MoHW), is reporting an average survival rate of 5 years after oral cancer has been diagnosed. This has not much improved in the past 20 years [4]. OSCC usually involves multistep carcinogenesis. Due to the large area that has been exposed to carcinogens, multiple lesions, also known as field cancerization, may develop at the same time at different neoplastic stages. This might be the reason for the high recurrence of OSCC after treatment [5]. Therefore, specific and sensitive biomarkers are highly desired for patients with OSCC and for individuals which have a high risk for

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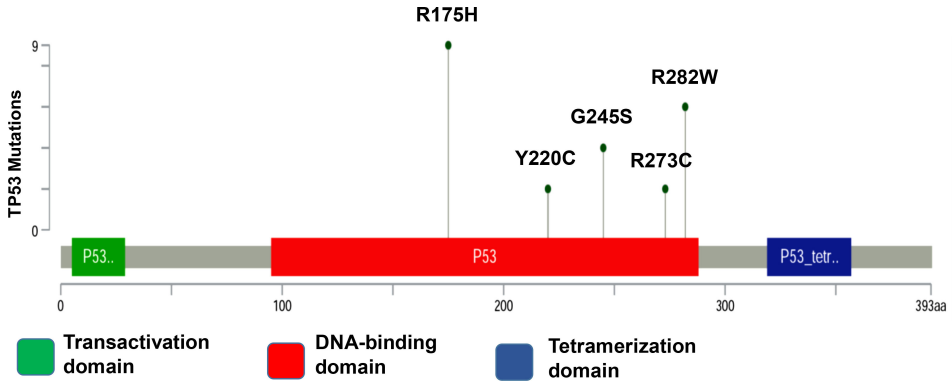

Fig. 1. TP53 mutations in patients with OSCC. Distribution of hotspot target mutations in the TP53 coding sequence. Mutation types are indicated by colored ovals, colored boxes are indicating domains.

developing OSCC [6]. Still, molecular biomarkers for guiding adequate diagnosis and management decisions have not been identified. In OSCC, TP53 is the gene with the highest mutation rate (65%–85%) [7], and the mutations in TP53 have predictive significance for the response to chemotherapy based on platinum complexes [8]. TP53 is a transcription factor that works as a tumor suppressor. TP53 is the most commonly mutated gene in various human cancers. In many types of cancer, the functions of the wild-type (WT) p53 protein are disturbed by these mutations [9]. The p53 pathway plays a critical role in the tumorigenesis and progression of HNSCC [10–12].

In cancerous cells, mutations in the TP53 gene frequently are found in its DNA-binding domain (DBD) between amino-acid residues 102 and 292 (out of 393 in total) [13]. Approximately 10% of these mutations result in the loss of function of p53 when no protein is synthesized due to nonsense or frameshift mutations or due to deletions. The remainder includes missense mutations, which result in a dysfunctional protein. Loss of function mutations include mutations that impair the ability of p53 to recognize its specific DNA sequence motifs [13–16].

Of 190 mutation sites, the most common 10 that are located in the DBD are found in approximately 30% of all missense mutations in HNSCC. This apparent preference for some mutations may be the result of the selection process that favors alleles that are coding for proteins whose structures and possibly functions do maximally contribute to a cancerous phenotype [17]. Recent developments in molecular diagnostics foster the use of blood-based genetic biomarkers for the diagnosis of various cancer types [18]. Cell-free DNA (cfDNA) is found in healthy people and patients with benign or malign tumors. It may originate from normal cells, including healthy leukocytes that undergo apoptosis, and also may be shed from dead cells, from healthy or cancerous tissue [19,20]. cfDNA has gained attention as a potential biomarker in oncology [21]. Cell-free circulating tumor DNA (ctDNA) may be released into the bloodstream by necrotic and apoptotic tumor cells and contains tumor-specific mutations [22]. These mutations may be detected in the blood of cancer patients by a simple blood sampling. This method has been termed “liquid biopsy” [23]. For head and neck cancer, so far studies have mainly focused on actionable oncogenic mutations, such as PIK3CA and HRAS, hotspot TP53 mutations, and also on human papillomavirus (HPV)-related biomarkers, which may serve as prognostic or predictive markers, in order to establish and to modify targeted therapy [20,24–28]. However, the accurate detection of ctDNA in plasma is a challenge, as the concentration of ctDNA may be low. This may greatly impair the reliable and valid assessment of tumor dynamics. Droplet digital polymerase chain reaction (ddPCR) is a method for performing digital PCR that is based on water-oil emulsion droplet technology. The DNA sample is fractionated into about 20,000 water-in-oil droplets, and PCR amplification of the template molecules occurs in each individual droplet [29,30]. High-copy templates and background are diluted, effectively enriching template concentration in target-positive partitions, allowing for the sensitive detection of rare targets especially in the cfDNA. In this study, we used ddPCR as a method to evaluate the five most frequent coding TP53 mutations, namely R175H, R282W, G245S, Y220C, and R273C, by using the OSCC patients cancerous tissue and cfDNA database (Fig. 1A) [17]. Clinicopathogenic variants were analyzed using the target mutation sites of OSCC patients.

Table 1
TP53 ddPCR-specific PrimePCR ddPCR mutation assays

TP53 primer/probe	Unique assay ID	Fluorophore	Amplicon length	Restriction enzyme
p.R175H	dHsaMDV2010105	FAM + HEX	65	HaeIII
p.Y220C	dHsaMDV2510536	FAM + HEX	64	HaeIII
p.G245S	dHsaMDV2516746	FAM + HEX	65	MseI
p.R273C	dHsaMDV2510538	FAM + HEX	65	HaeIII
p.R282W	dHsaMDV2516902	FAM + HEX	64	HaeIII

2. Materials and methods

2.1. Patients

In this study, we evaluated 107 patients with OSCC and 50 matched healthy controls. Tumor samples were obtained from OSCC patients during surgery. Before the surgery, none of the patients had received adjuvant chemotherapy or radiotherapy. Informed written consent was obtained from all participants. The Institutional Review Board (IRB) of Mackay Memorial Hospital has approved the study. IRB approval numbers are 12MMHIS178 and 15MMHIS104.

Cells were isolated from tissue sections by laser capture microdissection, following established protocols. DNA was extracted from cancerous tissue as previously reported [31]. 10 mL of whole blood were collected from the patients in the morning after fasting and before the surgery. Vacutainer blood collection tubes containing EDTA as anticoagulant (Becton Dickson, Franklin Lakes, NJ, USA) were used.

2.2. cfDNA extraction

The procedure for cfDNA extraction has been previously described [32,33]. In brief, plasma samples were spun at 1600 g for 15 min, the supernatant then was centrifuged at 11,000 g for 10 min. Subsequently, the samples were stored in 1 mL aliquots at $-80°$C until analysis. The workup of the plasma samples was done within 3 hours of blood collection. cfDNA was extracted from plasma samples with the CatchGene Catch-cfDNA Serum/Plasma kit (CatchGene Co. Ltd, New Taipei City, Taiwan) in accordance with the manufacturer's instructions and eluted into 30 μL of elution buffer, which was included in the kit. The cfDNA then was quantified with a Qubit 2.0 Fluorometer, using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, OR, USA).

2.3. Plasma DNA quantification

For sizing and quantifying the purified plasma DNA, a TapeStation 2200 (Agilent Technology, USA) with a high-sensitivity D1000 ScreenTape system (Agilent Technologies, USA) was used [33].

2.4. Droplet digital polymerase chain reaction

The plasma and tissue samples from all 107 cancer patients were analyzed for TP53 point mutations which had been identified in the primary tumor tissue by next-generation sequencing (NGS). Mutant-type (MT) and wild-type (WT) TP53 sequences were used as DNA templates for designing the ddPCR experiments (Bio-Rad Laboratories, CA, USA) assays in accordance with the MIQE guidelines [34]. ddPCR reactions were carried out in a volume of 20 μL, consisting of 10 μL of 2 $\times$ ddPCR Supermix for Probes (without dUTP), 1 μL of the five target-specific PrimePCR ddPCR Mutation Assays primer/probe mix for both mutant and wild type TP53 (Table 1), 0.5 μL restriction enzyme, and 40 ng of amplified cfDNA sample from the patients' plasma. For the NTC cell, purified water was used. 20 μL of a PCR reaction mixture was used for the generation of droplets. ddPCR was performed in a QX200 ddPCR system, following the manufacturer's instructions (Bio-Rad Laboratories). Based on the clearest separation of negative and positive droplet clusters, the thermal cycling conditions for all 5 assays were set to 95°C for 10 min (1 cycle), 94°C for 30 s and 55°C for 60 s (40 cycles), 98°C for 10 min (1 cycle), and 4°C indefinitely. To ensure experiment quality, wells with less than 10,000 droplets were considered invalid and excluded from the analysis. Positive controls served to verify assay performance and to facilitate the thresholding of the fluorescence values. In addition, positive controls were validated by comparing fractional abundance (FA) in FFPE samples with NGS mutation frequencies. The rate of false positives was estimated in five experiments for each assay using wild type only samples, where the total numbers of detected mutant (positive) droplets were used to set the thresholds, above which positive droplets in the patient samples were considered as true positives. ddPCR data were analyzed with the QuantaSoft package, v1.7.4.0917 (Bio-Rad Laboratories).

2.5. Validation of point mutations

We confirmed the identified somatic mutations by Sanger sequencing. For each point mutation, primer sets

Table 2
TP53 mutation sites for Sanger sequencing

TP53 mutation site		Sequence	TM (°C)	bp
p.R175H	Forward	GTGCAGCTGT-GGGTTGATT	58	223
	Reverse	GGGCCAGACC-TAAGAGCAAT		
p.Y220C	Forward	GCCCCTCCTC-AGCATCTTAT	58	237
	Reverse	TTGCACATCTC-ATGGGGTTA		
p.G245S	Forward	TGCTTGCCAC-AGGTCTCC	58	236
	Reverse	GGTCAGAGGC-AAGCAGAGG		
p.R273C/ p.R282W	Forward	GGGAGTAGAT-GGAGCCTGGT	58	248
	Reverse	GCTTCTTGTCC-TGCTTGCTT		

Table 3
Clinicopathological parameters with cfDNA concentration in patients with OSCC

	OSCC			C control		
Variables	N	Mean ± SEM	P	N	Mean ± SEM	p
Gender						
Male	96	53.90 ± 7.10	0.851	43	22.64 ± 3.35	0.9
Female	11	41.25 ± 13.71		7	32.04 ± 12.36	
Tumor size						
T1–T2	24	64.57 ± 7.69	0.024*			
T3–T4	83	106.9 ± 14.60				
Nodal stage						
N0	67	46.45 ± 5.69	0.009**			
N+	40	127.4 ± 20.32				
Stage						
I–II	21	48.58 ± 10.30	0.001**			
III–IV	86	96.51 ± 12.96				
TP53 mutation						
No	84	55.03 ± 7.96	0.2			
Yes	23	81.24 ± 27.92				

Student's t test. *Statistically significant ($p < 0.05$).

were designed in Primer3 version 0.4.0 (http://primer3.wi.mit.edu/), they are shown in Table 2. For Sanger sequencing, PCRs were performed using a standard hot start kit. Amplicons then were sequenced with the ABI BigDye Terminator Cycle Sequencing kit on an ABI 3730xl DNA analyzer instrument (Applied Biosystems, Foster City, CA, USA).

2.6. Statistical analysis

For all statistics, the Prism 5 package (GraphPad, San Diego, CA, USA) and SPSS 18.0 (SPSS Inc, Chicago, IL) were used. Mann-Whitney, Fisher, and Kruskal-Wallis tests were used to compare differences among various variants. Their influence on survival was assessed with a Kaplan-Meier analysis. Differences were considered statistically significant for p-values of < 0.05, < 0.01, or < 0.001. Cross comparisons that had no significant differences have not been marked.

3. Results

In this study, 107 patients with OSCC and 50 matched healthy controls were investigated. The distribution of cfDNA sizes was similar between OSCC patients and healthy donors, the average size of cfDNA was 150–200 bp (Fig. S1). Table 3 shows the clinical characteristics of the study participants. Preoperative cfDNA plasma concentrations were between 6.1 and 646 ng/mL (Fig. 2A). in OSCC, the mean concentration of cfDNA was 71.9 ± 8.3 ng/mL, in the control group it was 11.9 ± 1.6 ng/mL. Several clinicopathological parameters were analyzed in this study: There was no association of the cfDNA concentration with age, sex, perineural invasion, or cell differentiation (data not shown). However, cfDNA levels correlated with tumor size (Fig. 2B) and TNM staging (Fig. 2C). Plasma cfDNA levels were higher in tumor patients with neck lymph node metastasis compared to those without neck lymph node metastasis (Fig. 2D). Patients with TP53 mutations had no significantly higher cfDNA concentration than those without a TP53 mutation (Fig. 2E); this finding was similar to that of a previous study [33].

All 107 patients were assessed for TP53 mutations by performing ddPCR of primary cancerous tissue and preoperative cfDNA. Five hotspot target mutations were identified in this study. Five assays each were run to detect p.R282W, p.R175H, p.R273C, p.Y220C, and p.G245S mutations through ddPCR. PCR for wild type alleles was run in parallel, and samples were considered to have a mutated TP53 when mutations were detected at an FA of $> 1\%$. In total, 23 patients (21.5%) had cancerous tissue with mutations in the five targeted points (R175H: 9, R282W: 6, G245S: 4, Y220C: 2, and R273C: 2; Fig. 3). In no patient, more than one TP53 hotspot mutation was encountered. MT-TP53 was not associated with tumor size, sex, age, cellular differentiation, or perineural invasion (Table 4). Patients with OSCC and DM had a tendency for mutations of TP53, but this correlation was statistically not significant. When neck lymph node metastasis was present in patients, the probability of having a mutated TP53 was greater than in those without neck lymph node metasta-

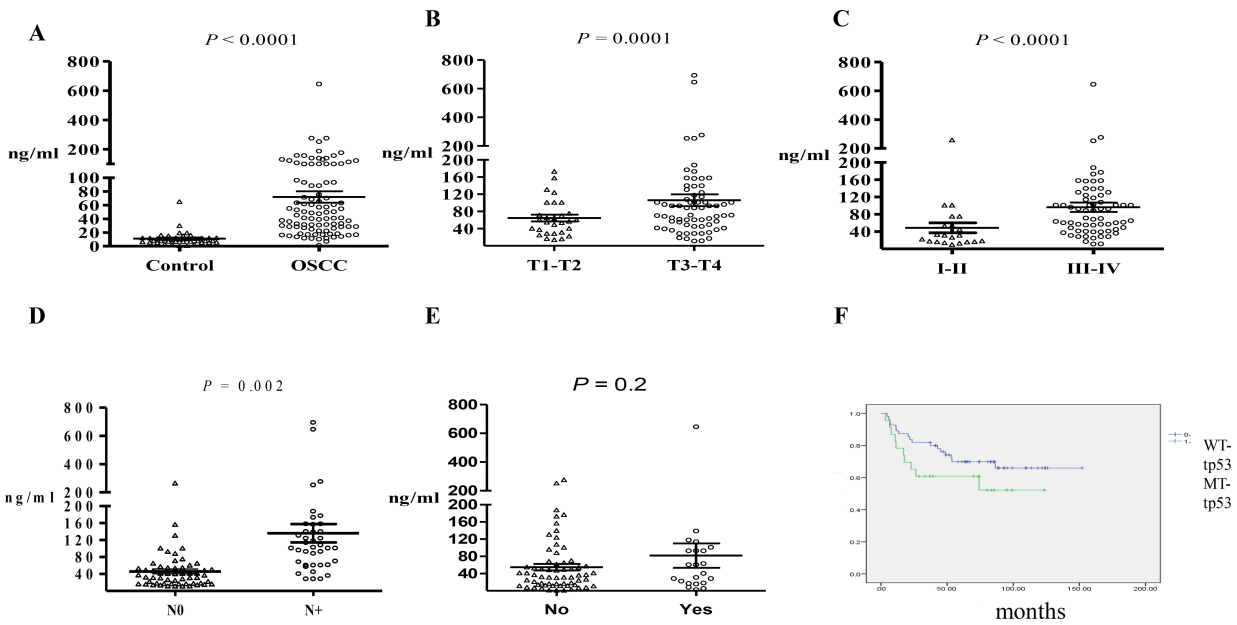

Fig. 2. Comparison of cfDNA plasma levels in (A) healthy controls versus patients with OSCC preoperatively; (B) patients with different tumor sizes preoperatively; (C) patients without metastasis in the neck lymph nodes; (D) patients with early- and late-stage carcinoma; and (E) patients with different statuses of lymphovascular invasion. (F) Kaplan-Meier analysis of patients with MT-TP53 and WT-TP53. Scatter plots displaying mean ± standard deviation were calculated by the Mann-Whitney test.

Fig. 3. Clinicopathological parameters of OSCC. Colored boxes indicate different parameters and patients with MT-TP53.

sis (Table 4). Patients with MT-TP53 exhibited a trend to poor overall survival rate compared with those with WT-TP53 (Table 4 and Fig. 2F).

On the basis of the cancerous tissue, matched cfDNA samples were tested for the five TP53 target hotspot mutations by using ddPCR (Figs 4–6). In the circulating DNA however, identifying copies of mutant DNA with confidence was not possible in all analyzed cases (Fig. 3). A few samples with MT-TP53 had a limited number of droplets and fractional mutations below the minimum value which was predicted by the Poisson distribution analysis. In WT-TP53 background templates, we found mutations yielding FAs as low as 0.01%. In OSCC, cfDNA shedding was relatively low, and the allele frequencies of the mutations were low, too. Not in all patients where MT-TP53 was found in cancerous tis-

Table 4
Clinicopathological parameters with TP53 hotspot target mutations

TP53 mutation	Cancerous tissue			cfDNA		
	MT-TP53	WT-TP53	p	MT-P53	WT-TP53	p
Gender						
Male	21	75		12	84	
Female	2	9	1	1	10	1
Average age (y/o) site	55.8	56.6	0.90	55.5	56.8	0.9
Buccal	6	30		4	33	
Tongue	7	14		3	18	
Gingiva	9	23		5	27	
Retromolar	0	3		0	3	
Mouth floor	0	3		0	3	
Lip	1	2		1	2	
Other	0	9	0.28	0	9	0.6
Tumor size						
T1–2	6	18		1	23	
T3–4	17	66	0.77	12	71	0.3
Pathological stage						
Stage 1–2	6	15		1	20	
Stage 3–4	17	69	0.38	12	74	0.4
lymph node metastasis						
N0	10	57		4	63	
N1–3	13	27	0.03*	9	31	0.01*
ENE						
Positive	5	12		3	14	
Negative	18	72	0.51	10	80	0.4
Perineural invasion						
Positive	8	26		5	29	
Negative	15	58	0.80	8	65	0.7
Differentiation						
Well	13	46		7	52	
Moderate	9	33		5	37	
Poor	1	5	0.59	1	5	0.9
Lymphovascular invasion						
Positive	5	15		3	17	
Negative	18	69	0.76	10	77	0.7
DM						
Yes	13	31		7	37	
No	10	53	0.09	6	57	0.3
Recurrence						
Positive	4	22		2	24	
Negative	19	62	0.26	11	70	0.7
Status						
Expired	12	28		6	34	
Alive	11	56	0.09	7	60	0.5

Fisher's exact test. *Statistically significant ($p < 0.05$).

sue had detectable amounts of cfDNA. Of these 23 patients, 13 (56.5%) had one of the target TP53 mutations. Six, three, two and one patients had R175H, R282W, G245S, and Y220C mutations, respectively. We could not confidently identify copies of mutant TP53-DNA with the R273C mutation in the circulating DNA (Table 5 and Fig. 3). In the comparison with clinicopathological parameters, cfDNA with TP53 hotspot target mutations was related to lymph node metastasis (Table 4). These targeted mutations were validated through Sanger sequencing in all cancerous tissue samples but could not be detected in cfDNA (Figs 4–6).

4. Discussion

Liquid biopsies may be used for the analysis of ctDNA, as recent research has shown. There is a wide

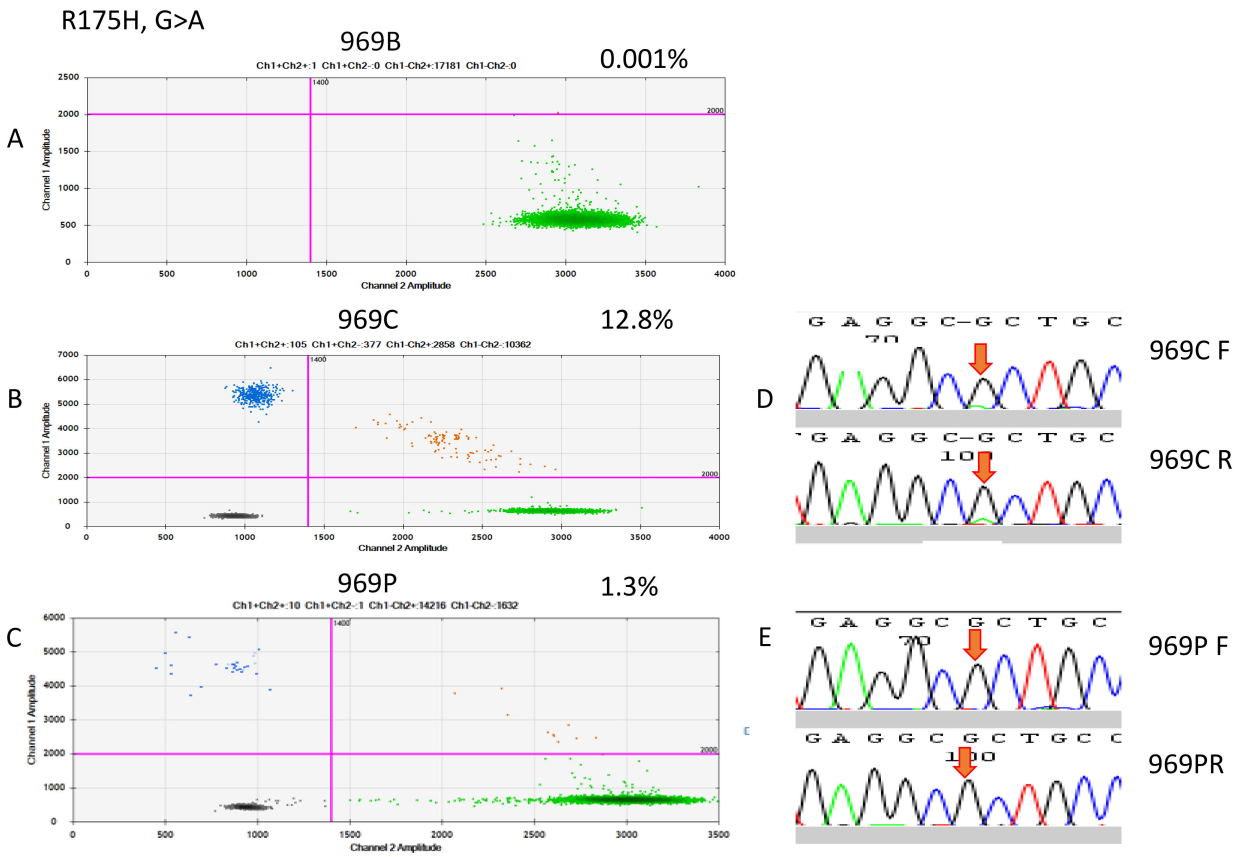

Fig. 4. Two-dimensional plot, showing the distribution of the droplet amounts of mutant MT-R175H in ddPCR of patient 969. (A) Merged ddPCR results in the duplicates of corresponding blood samples from patient 969. Blue dots represent MT-positive droplet clusters, dark gray dots represent negative droplet clusters, and orange dots represent MT/WT-positive droplets. WT-positive droplets are represented by green dots, demonstrating that cfDNA was present in the samples and that the ddPCR conditions were satisfactory. The purple lines are the thresholds that have been manually placed, in order to distinguish positive and negative droplets. They were set at fluorescence values based on the ddPCR data of (B) FFPE samples and (C) cfDNA samples. (D) Sanger sequencing indicated a G-to-A point mutation in the FFPE sample, but it was not detectable in the cfDNA sample.

Table 5
MT-TP53 in cancerous tissue and cfDNA of patients with OSCC

Mutation point	Cancerous tissue	cfDNA	Blood DNA	Correlation rate
R175H	9	6	0	77.7%
R282W	6	3	0	50.0%
G245S	4	2	0	50.0%
Y220C	2	1	0	50.0%
R273C	2	0	0	0
Total	23	13	0	56.5%

range of potential applications, like early diagnosis, personalized treatment, and the prediction of disease progression [35]. Early cancer diagnosis and disease surveillance in precision medicine request to research the role that ctDNA is playing in OSCC. ctDNA is present in early cancers [36]. However, tumor fractions which are shedding detectable amounts of ctDNA are not yet well studied in respect to tumor type and stage [37]. In different malignancies, ctDNA has been widely reported, but it has been studied not much yet in OSCC. Tumor specific TP53 mutations in low levels of ctDNA from HNSCC have been reported by Ginkel et al. [38,39]. However, only six cases of HNSCC were tested using ddPCR [39]. This study is, to the best of our knowledge, the first study that evaluates the presence of ctDNA in patients with OSCC through the detection of multiple target TP53 point mutations by ddPCR, requiring the use of very sensitive detection methods. Our data reveals that cancerous tissue and cfDNA are not associated. Only 56.5% of cases had matched target TP53 mutations between ctDNA and tumor DNA. However, the fraction of tumors that are shedding ctDNA at detectable levels, based on tumor type and stage, may be the reason for the discrepancy in MT-TP53 levels between tissue DNA and cfDNA [37]. In gastrointesti-

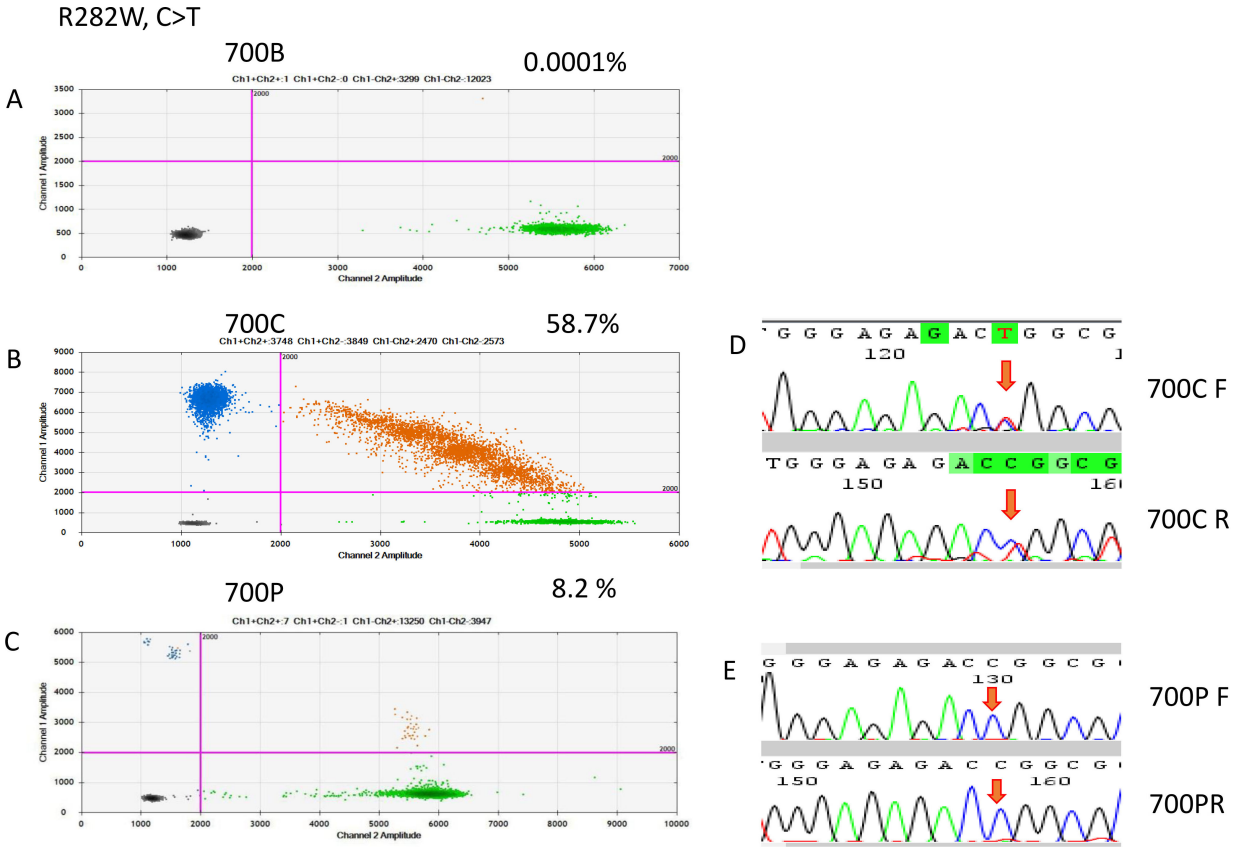

Fig. 5. ddRT-PCR results of patient 700. The two-dimensional plots show the amount of MT-R282W droplets in the analysis. (A) Blood sample, (B) FFPE sample, and (C) cfDNA sample. (D) Sanger sequencing indicated a C-to-T point mutation in the FFPE sample, but it was not detectable in the cfDNA sample.

nal stromal tumors, ctDNA shedding has been found to be relatively low, and also the allele frequencies of the mutations were low [40]. Only in 1 of the 13 cfDNA-matched samples ctDNA was detectable, the allele fraction was 12.3%. In a ctDNA screening experiment in hepatoma, negative results were observed in about 50% of the patients [41]. In our data, 56.5% of OSCC samples were found to have TP53 target mutations that are typical for cancerous tissues. This result might have been caused by the cancer type and by the tumor microenvironment, which are different in every patient, as well as effects of the immune system which are affecting the evolution and the lethality of a tumor. In contrast to infectious diseases, which are characterized by well-defined causative agents of highly conserved biology, cancer presents as a heterogeneous collection of many diseases, each of which is adding heterogeneity [37].

In particular, the low abundance of ctDNA and CTCs is limiting the usefulness of liquid biopsies in the early stages of cancer, and drawing large blood volumes in order to obtain more genetic material for the analysis is not always possible for clinical reasons. In cancer patients, the plasma concentration of cfDNA is higher than in healthy subjects, and it is increasing with the progression of the cancer. In healthy individuals, its blood concentration is approximately 13 ng/mL; in patients with metastatic cancer, up to 180 ng/mL have been reported [22,42]. Most cfDNA consists of small linear fragments with a length of a few hundred base pairs. Its concentration is varying significantly between patients. In a previous study, the mean concentration of cfDNA in OSCC was found to be 53.1 ng/mL, in the control group it was less than half (24.0 ng/mL) [33]. For the efficient, non-selective amplification of small linear DNA fragments, T oligo-primed PCR was chosen. In accordance with modern molecular cloning strategies for NGS, which normally add an “A” to the 3′ terminus of the target DNA, we adopted a homogeneous double-stranded half adaptor (HA), which is formed by annealing a “P oligo” with a “T oligo” at room temperature. P oligo is phosphorylated (the phosphate group is required by the ligase when ligating HA to the target DNA) at

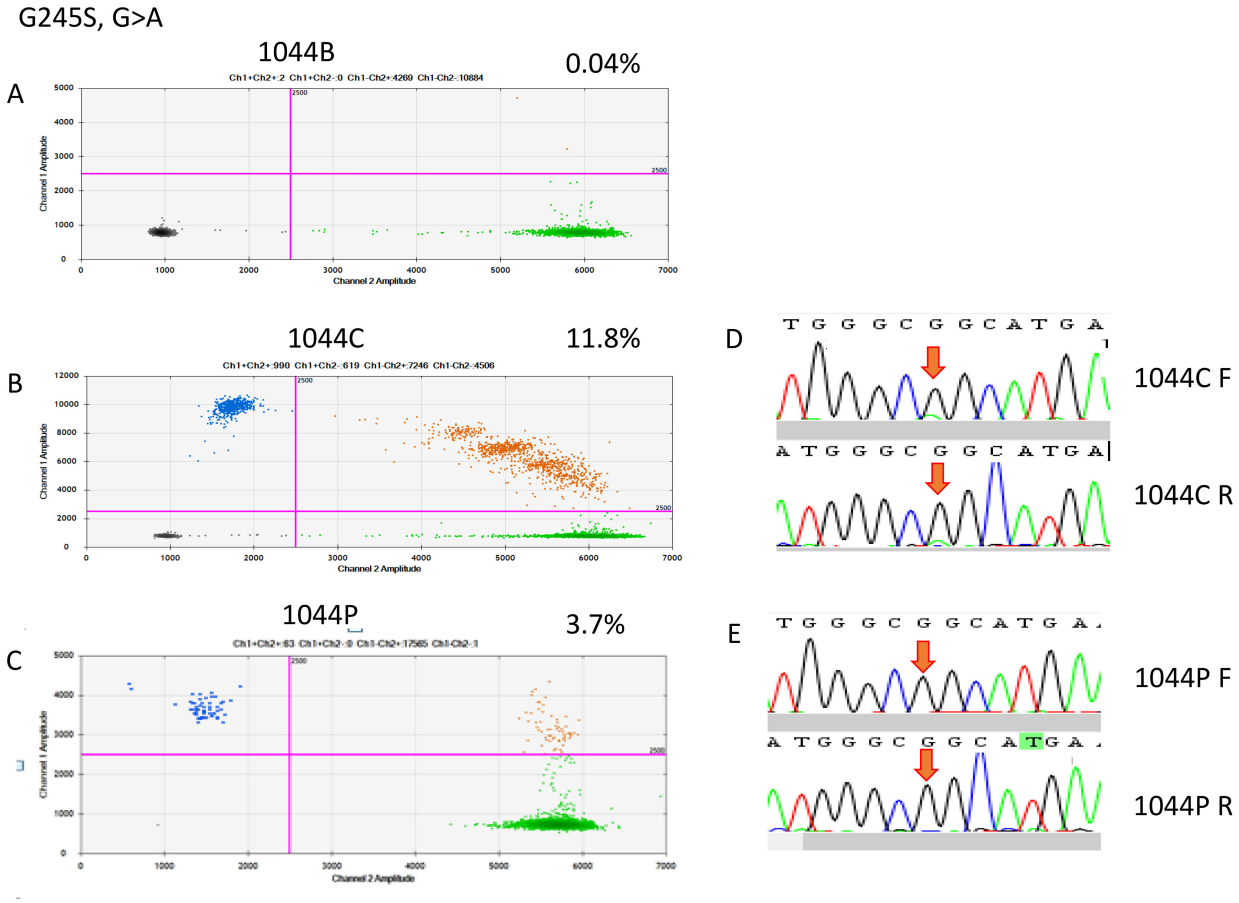

Fig. 6. ddRT-PCR results of patient 1044. The two-dimensional plots show the amount of MT-G245S droplets in the analysis. (A) Blood sample, (B) FFPE sample, and (C) cfDNA sample; (D) Sanger sequencing indicated a G-to-A point mutation in the FFPE sample, but it was not detectable in the cfDNA sample.

the 5′ end, whereas T oligo carries an additional T at the 3′ end to enable a sticky-end ligation to the target DNA fragments [43]. Originally, the HA structure was designed for the construction of multiplex bar coded paired-end ditag sequencing libraries (US 8481699 and US 8829172). This design prevents the self-ligation of the adaptors and allowed us to maximize the ligation efficiency to an unprecedented level. Adding markers for CTCs and the use of implanted devices with materials that are able to bind ctDNA [44] or assays that can simultaneously test for multiple mutations in the same reaction [45]. may increase the amount of detected CTCs/ctDNA. Furthermore, successful cfDNA detection requires the selection of tumor-specific gene aberrations. This would be necessary for NGS-based methods. However, with ddPCR-based methods only a limited number of mutations may be analyzed in a single reaction. Digital droplet PCR offers a platform that may easily be used in a clinic, it is highly sensitive and offers a rapid turnover [46]. Regarding the application of ddPCR in a clinical environment, as much DNA needs to be collected as possible from liquid biopsies, such as plasma or Pap samples. Wang et al., evaluated a Tao brush for sampling in close proximity to tumors [47]. and found an improvement of the detection limit. However, to successfully implement a diagnostic test for screening, the sampling procedure must not impose too much stress on the patient [48].

Although the sensitivity of ddPCR is high, one shortcoming is the limited number of mutations that may be detected in a single DNA sample of low abundance. The multiplexing of ddPCR assays may allow the simultaneous screening of several mutations [49]. This approach recently proved efficient for the genotyping of KRAS mutations in non-small-cell lung cancer [50]. and may further increase the usefulness of screening TP53 for mutations, because the mutations of interest are spanning the entire TP53 gene [51]. Therefore,

hotspot mutations are a preferred target for the screening cfDNA by ddPCR. TP53 is the most commonly mutated gene in OSCC as reported by the Cancer Genome Atlas 2015 [12]. Most TP53 mutations are found in the DNA binding domain, they effectively block TP53 from binding to the transcription factor responsive elements and inhibit the transactivation of the target genes downstream of the binding site. R175H, R282W, G245S, Y220C, and R273C are the five most frequently mutated amino acids of p53 in HNSCC. They all are located in its DBD [12]. In this study, it was not clear whether our ddPCR strategy achieved comparable sensitivity in detecting ctDNA in OSCC patients. In addition, we were only able to select five mutations for the screening of cfDNA, and the results were negative in about half of the patients. Multiplex ddPCR therefore might be a feasible solution.

Regarding the association between TP53 mutations and metastasis in lymph nodes, the findings in this study have been inconsistent [8,52–55]. In HPV, DNA-negative HNSCC with TP53 mutations that were disrupting the protein function was independently associated with metastasis in lymph nodes [56]. In our study, target TP53 mutations were located on the DNA-binding site. Tumors with TP53 mutations at the surface of TP53 that is establishing the contact with the DNA (L2, L3 + LSH) were more aggressive than tumors with mutations of TP53 that were outside these regions [57]. Notably, patients with tumors where TP53 is mutated in L3 and LSH have a poorer prognosis of survival, and in tumors with TP53 mutations in domains L2 and L3 + LSH, more frequent relapses and less disease-free time were observed in comparison with other tumors, implying an aggressive phenotype [57]. Similar observations have been reported for gastric cancer [58,59] and colorectal carcinoma [59]. Because our target mutations are located on the DNA-binding site, they might be associated with lymph node metastasis.

5. Conclusion

TP53 target somatic mutations can be detected with ddPCR in OSCC. Low levels of cfDNA and different cancer cell types may lead to diverse results between cancerous tissue and cfDNA analyses. Future research on cfDNA may quantify diagnostic biomarkers in the surveillance of these patients. The clinical significance of analyzing mutations in cfDNA samples as a diagnostic and predictive biomarker in patients with OSCC needs to be evaluated further. More studies should be performed with the goal to improve the sensitivity of the assays by targeting multiple mutations and determining the extraction of cfDNA from larger sample sizes and evaluating the usefulness of repeated, sequential, blood sampling.

Acknowledgments

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Author contributions

Conception: CJ Liu.
Lab work: HW Cheng.
Interpretation or analysis of data: LH Lin, CJ Liu.
Preparation of the manuscript: LH Lin, CJ Liu.
Revision for important intellectual content: CJ Liu.
Supervision: CJ Liu.
All authors have approved the final version of the manuscript and agree to be accountable for it.

Conflict of interest

The authors declare no potential conflicts of interest.

Supplementary data

The supplementary files are available to download from http://dx.doi.org/10.3233/CBM-210275.

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