Endocrine (2019) 64:614–621
https://doi.org/10.1007/s12020-019-01877-1

ORIGINAL ARTICLE

Clonal analysis of early-stage bilateral papillary thyroid cancer identifies field cancerization

Xingyun Su[1] · Shitu Chen[1] · Kuifeng He[1] · Zhuochao Mao[1] · Jiaying Ruan[1] · Jie Zhou[2] · Xiaodong Teng[2] · Judy Jin[3] · Thomas J. Fahey III[4] · Weibin Wang[1] · Lisong Teng[1]

Received: 5 December 2018 / Accepted: 18 February 2019 / Published online: 26 February 2019

Abstract

Introduction Bilaterality is a newly identified indicator for aggressive tumor behavior and poor outcome in papillary thyroid cancer. However, the clonal origin of these bilateral tumors remains unclear.

Methods Here we analyzed 28 pairs of early-stage papillary thyroid cancers (stage I–II without extra-thyroidal extension, lymph node metastasis or distant metastasis) that underwent surgery at First Affiliated Hospital of Zhejiang University School of Medicine (Hangzhou, China). Genomic DNA was extracted from paraffin-embedded tissues after microdissection and analyzed for *BRAF* mutation and X-chromosome inactivation.

Results A total of 16 patients (16/28, 57.1%) harbored different *BRAF* status in bilateral tumors. Fourteen patients were available for X-chromosome inactivation assay and 10 of them achieved informative results. Bilateral tumors from four cases had distinct patterns of X-chromosome inactivation. Combining the results of X-chromosome inactivation and *BRAF* analysis, we demonstrated that at least 64.3% (18/28) cases harbored discordant X-chromosome inactivation or *BRAF* status, indicating their independent clonal origin in bilateral tumors.

Conclusions The present study confirms "field cancerization" in early-stage bilateral thyroid cancers, suggesting that these subtype papillary thyroid cancers should be treated as independent and localized tumors.

Keywords Papillary thyroid cancer · Bilaterality · Early stage · Clonal origin · Field cancerization

Introduction

The incidence of thyroid cancer has increased rapidly over the past few decades [1, 2]. Papillary thyroid cancer (PTC) is the most common type and highly curable after standard treatment including surgery with/without radioactive iodine [2–4]. Bilaterality is a characteristic present in 13–56% thyroidectomy cases [5–7]. Previous studies from our group have demonstrated that bilateral PTCs showed more aggressive behaviors, advanced tumor stage and worse outcome when compared to unilateral, and even unilateral-multifocal PTCs [8, 9]. Therefore, bilaterality is an adverse factor which should be considered in risk stratification, treatment and subsequent follow-up of thyroid cancer. However, it remains unclear whether the contralateral tumors arise independently (independent clonal origin) or result from intraglandular metastasis (same clonal origin).

These authors contributed equally: Xingyun Su, Shitu Chen

✉ Weibin Wang
wbwang@zju.edu.cn

✉ Lisong Teng
lsteng@zju.edu.cn

[1] Cancer Center, First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang, China

[2] Department of Pathology, First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang, China

[3] Department of Endocrine Surgery, Cleveland Clinic, Cleveland, OH, USA

[4] Department of Surgery, New York Presbyterian Hospital and Weill Medical College of Cornell University, New York, NY, USA

The answer to this question will not only improve the understanding of tumorigenesis, but also guide clinical management since the treatment of localized and metastatic tumor is substantially different.

Advances in molecular biology provide more specific and reliable approaches to clonal analysis. Lyon et al. found either paternal or maternal X chromosome would be randomly inactivated by methylation during early stage of embryogenesis in female [10]. Since the methylation status can be stably inherited by progenitor cells, tumors originate from a single cell will present the same methylation phenotype [10]. Thus, X-chromosome inactivation (XCI) assessment is the gold standard for clonal analysis. What's more, transformation and progression of cancer is accompanied with numbers of genetic alterations, and most of them are passenger mutations not shared by all foci even if they are developed from the same clone [11, 12]. In contrast, driver mutations are the trigger of carcinogenesis and retained homogenously during clonal expansion, which can serve as markers to discriminate clonal origin of tumors [11, 13]. In thyroid cancer, *BRAF* mutation is regarded as an early molecular event and good clonal marker [14], which could induce the occurrence of goiter and PTC in transgenic mice [15].

The clonal origin of multifocal thyroid cancer is a long-standing controversy. Some researchers support the independent clonal origin, that is, the lesions are developed independently from separate origins in the same environmental background [16–18]. While others propose that lesions arise from a single clone and the tumor clones spread through intraglandular metastasis, giving rise to distinct but genetically related metastatic lesions [19, 20]. So far, few research has specifically focused on the clonal origin of bilateral thyroid cancer. Our previous study initially aimed to identify the clonal origin of contralateral tumor and metastatic lymph node in bilateral PTCs, in which nearly all the patients had advanced stages with extra-thyroidal extension, lymph node metastases or distant metastases [19]. We found that contralateral lesion and metastatic lymph node usually shared concordant clonal origin with primary tumor, suggesting the unnegligible role of intrathyroidal metastasis [19].

With the improvement of detection technologies, more and more thyroid cancers are diagnosed in early stage [21–23]. Over-diagnosis and over-treatment are not rare in clinical practice, and many clinicians propose that unnecessary treatment should be avoided in early-stage thyroid cancer which is precursor to a more aggressive process [23–25]. We hypothesize that intrathyroidal metastasis has not occurred in early-stage tumors, so that the clonal origin of early-stage bilateral thyroid cancer may be different from those with invasion and metastasis.

Materials and methods

Case selection and tumor specimen collection

Twenty-eight PTC patients were enrolled according to the following criteria: (1) bilateral tumors were diagnosed and treated with total thyroidectomy in the First Affiliated Hospital, Zhejiang University School of Medicine (Hangzhou, China); (2) all the tumor nodules were classical PTC; (3) tumors were in stage I–II without extra-thyroidal extension, lymph node metastasis or distant metastasis; (4) no history of irradiation or clinical presentation of Hashimoto's thyroiditis; (5) the bilateral tumors were available to be analyzed as a pair.

Pathological diagnoses were confirmed by three senior pathologists according to the World Health Organization Classification [26]. Tumor stage was classified according to 8th TNM system of American Joint Committee on Cancer (AJCC) [27]. This study was approved by the Institutional Review Board of First Affiliated Hospital, Zhejiang University School of Medicine. Informed consent has been obtained from each patient after full explanation of the purpose and nature of all procedures used according to the Helsinki Declaration of 1975, as revised in 1983.

Microdissection and DNA extraction

Genomic DNA was isolated from paraffin-embedded specimens. A 4-μm-thick section was stained with hematoxylin and eosin (H&E) as template for microdissection. Four to eight unstained 10-μm-thick sections were deparaffinized in xylene and rehydrated in graded ethanol. Tumor margins were marked by comparing with the H&E-stained slide and tumor tissues were then micro-dissected from unstained sections and transferred to 1.5-mL Eppendorf tubes. DNA was released in 500-μL tissue lysis buffer (10 mmol/L Tris, 0.2 mmol/L EDTA and 0.5% sodium dodecyl sulfate; pH 8.0) plus 0.1 mg proteinase K at 56 °C overnight, and then added Chelex-100 resin (Sigma) and incubated at 37 °C for 1 h to improve the quality of DNA. Finally, genomic DNA was extracted by phenol/chloroform and precipitated in ethanol. Concentration and purity of genomic DNA were evaluated by spectrometry.

X-chromosome inactivation assessment

The human androgen receptor gene (HUMARA) in X chromosome has a highly polymorphic CAG repeat (~90%) and methylation-sensitive restriction enzyme sites for HhaI and HpaII [28, 29]. The detail conditions and primers used in HUMARA assay can be found in our previous study [19]. Allele intensity was determined by the peak height and

Table 1 Characteristics of the enrolled patients

Patient ID	Gender	Age at diagnosis (years)	Disease extent at surgery				Follow-up time (months)	Last known disease status
			Extra-thyroidal extension	Lymph node metastases	Distant metastases	Stage		
1	F	51	No	No	No	I	69	AFD
2	F	43	No	No	No	I	107	AFD
3	F	27	No	No	No	I	102	AFD
4	F	35	No	No	No	I	97	AFD
5	F	59	No	No	No	I	97	AFD
6	F	49	No	No	No	I	96	AWD
7	F	50	No	No	No	I	96	AFD
8	F	58	No	No	No	I	91	AWD
9	M	64	No	No	No	II	88	AFD
10	F	37	No	No	No	I	84	AFD
11	F	56	No	No	No	I	81	AFD
12	F	46	No	No	No	I	81	AFD
13	F	45	No	No	No	I	81	AFD
14	F	48	No	No	No	I	81	AFD
15	F	41	No	No	No	I	77	AFD
16	F	44	No	No	No	I	69	AFD
17	F	41	No	No	No	I	69	AFD
18	F	50	No	No	No	I	69	AFD
19	F	64	No	No	No	II	66	AFD
20	F	47	No	No	No	I	66	AFD
21	F	56	No	No	No	I	66	AFD
22	F	27	No	No	No	I	66	AFD
23	M	32	No	No	No	I	65	AFD
24	F	42	No	No	No	I	63	AFD
25	M	65	No	No	No	I	63	AFD
26	F	32	No	No	No	I	63	AFD
27	M	48	No	No	No	I	62	AFD
28	M	63	No	No	No	I	60	AFD

F female, *M* male, *AFD* alive free of disease, *AWD* alive with disease

modified to uniform the volume of size marker (Genescan-500Liz; ABI Perkin Elmer, Warrington, UK). The corrected allele ratio was calculated as follows: (peak 1 height of undigested sample ÷ peak 2 height of undigested sample ÷ Liz 200-bp height of undigested group)/(peak 1 height of digested sample ÷ peak 2 height of digested sample ÷ Liz 200-bp height of digested group). If the corrected ratio was less than 0.2 or more than 2, we considered the sample as nonrandom XCI [30]. Tumors were regarded as polyclonal if discordant inactivation patterns were detected.

BRAF mutation analysis

We amplified the exon 15 of *BRAF* gene using the primer sequences described in previous study [31]. Total volume of polymerase chain reaction (PCR) was 25 µL including 200 ng genomic DNA as template, 1 × PCR buffer with 2.0 mmol/L magnesium chloride, 0.2 mmol/L of each deoxynucleotide triphosphate and 1 unit of HotStart Taq polymerase (Takara, Shuzo, Japan). The reaction was conducted as follows: 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, and then a final extension at 72 °C for 10 min. A 224-base pair (bp) product was finally amplified. PCR products were visualized by electrophoresis in 2% agarose gel containing ethidium bromide (10 µg/mL) and sequenced by ABI PRISM 3730XL DNA Analyze (Applied Biosystems, Foster City, CA) using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA). The sequencing results were compared with the standard *BRAF* gene sequence (GenBank accession number: NC_000007.14).

Table 2 *BRAF* mutations in bilateral cases

Patient ID	Tumor left		Tumor right		Clonality
	Tumor size (mm)	Mutation	Tumor size (mm)	Mutation	
1	15	T1799A	16	T1799A	
2	25	WT	13	WT	
3	10	T1799A	12	WT	Independent
4	8	T1799A	5	WT	Independent
5	20	WT	10	WT	
6	18	T1799A	13	WT	Independent
7	10	T1799A	8	WT	Independent
8	8	WT	11	T1799A	Independent
9	25	WT	6	T1799A	Independent
10	9	T1799A	11	WT	Independent
11	16	T1799A	13	T1799A	
12	8	T1799A	8	WT	Independent
13	5	T1799A	15	WT	Independent
14	6	T1799A	8	T1799A	
15	8	T1799A	7	WT	Independent
16	6	WT	6	T1799A	Independent
17	6	WT	7	T1799A	Independent
18	9	T1799A	10	T1799A	
19	30	T1799A	6	T1799A	
20	15	T1799A	6	WT	Independent
21	12	T1799A	16	T1799A	
22	3	WT	9	T1799A	Independent
23	6	WT	7	WT	
24	8	WT	6	T1799A	Independent
25	5	T1799A	6	T1799A	
26	10	T1799A	5	T1799A	
27	10	WT	5	T1799A	Independent
28	15	T1799A	13	T1799A	

WT wild type

Table 3 X-chromosome inactivation (XCI) analysis in bilateral cases

Patient ID	Tumor location	XCI		Clonality
		Corrected allele ratio	Inactivated allele	
3	Left	0.19	S	Independent
	Right	7.3	L	
4	Left	0.13	S	
	Right	0.04	S	
5	Left	0.11	S	NA
	Right	1.24	Random	
6	Left	0.003	S	
	Right	0.0015	S	
7	Left	0.71	Random	NA
	Right	0.22	S	
8	Left	0.15	S	
	Right	0.29	S	
11	Left	2.61	L	Independent
	Right	0.12	S	
14	Left	0.25	S	Independent
	Right	72.3	L	
15	Left	0.49	S	Independent
	Right	13.3	L	
18	Left	0.54	Random	NA
	Right	9.2	L	
21	Left	0.008	S	
	Right	0.034	S	
22	Left	3.72	L	NA
	Right	0.61	Random	
24	Left	0.1	S	
	Right	0.14	S	
26	Left	3.5	L	
	Right	2.4	L	

S small allele, *L* large allele, *NA* non-available, the informative result is not available

Results

Characteristics of 28 bilateral PTC patients (23 females and 5 males) in this study were shown in Table 1. Patients' ages were ranged from 27 to 65 years old (mean 47 years). All patients were in the early stage (26 patients in stage I and 2 patients in stage II) and had no evidence of extra-thyroidal extension, lymph node metastasis or distant metastasis after surgery. At the time of this study, durations of follow-up ranged from 60 to 107 months (mean 77.7 months) and majority of patients (26/28) were alive free of disease (AFD) except two patients were alive with disease (AWD).

The *BRAF* mutation status were summarized in Table 2. A total of 16 patients (16/28, 57.1%) had different *BRAF* status in bilateral tumors, that was, *BRAF* mutation positive and negative tumors coexisted in both lobes of the same patient. We considered that contralateral tumors of these patients developed from independent neoplastic clones. On the other hand, 12 patients (12/28, 42.9%) showed concordant *BRAF* status in bilateral tumors including nine cases harbored *BRAF* mutation and three patients were wild type.

We performed XCI assessment in all the 23 female patients, but 6 patients (No. 1, 12, 13, 17, 19, 20) did not yield adequate DNA, 3 patients (No. 2, 10, 16) didn't harbor the polymorphism in HUMARA gene and 4 patients (No. 5, 7, 18, 22) presented as random results possibly because of the interference from normal cells. Finally, 10 pairs achieved informative results of XCI assessment (Table 3). The XCI assay results of patient No. 3 were shown in Fig. 1 as an example. Bilateral tumors from four cases were discordant in X-chromosome inactivation patterns indicating different clonal origin of contralateral tumor from primary lesion. The other six cases demonstrated concordant XCI patterns including five cases harbored inactivated X-chromosome in short allele and one case in long allele of the AR gene.

Combined results of XCI and *BRAF* analysis were shown in Table 4. A total of 18 patients (18/28, 64.3%) had

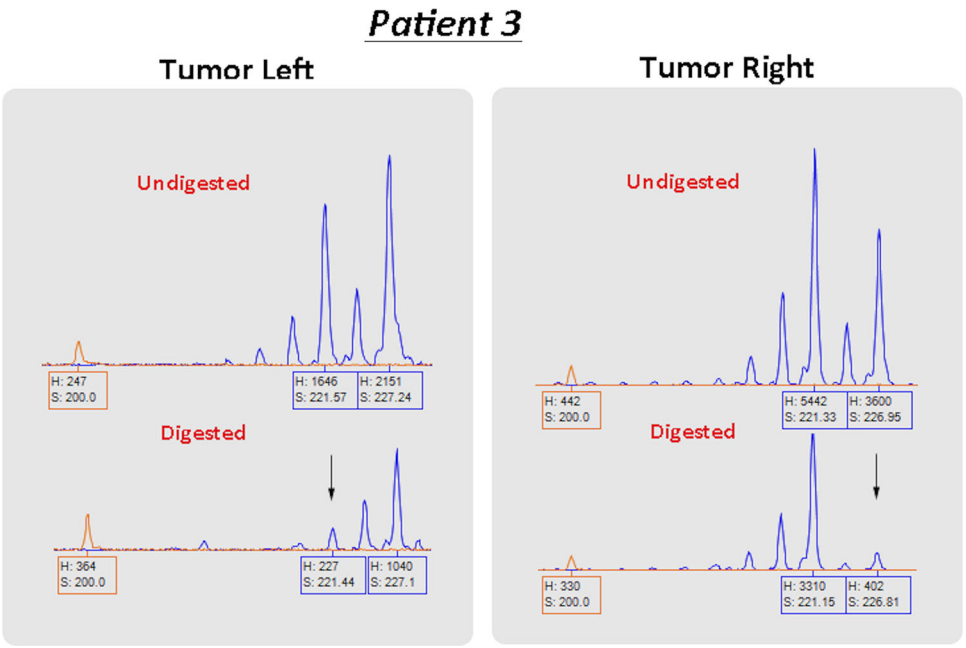

Fig. 1 Amplified DNA from tumor showed two alleles before digestion indicating the polymorphic CAG repeat in HUMARA gene. We found loss of different allele between left and right tumors (indicated by arrow) after digestion. Therefore, the bilateral tumors in Patient 3 had different patterns of allele inactivation and originated from independent cell clone. H height, S size

discordant XCI or *BRAF* status in bilateral tumors, indicating their contralateral tumors developed independently and should be regarded as a second tumor rather than metastatic site of the primary.

Discussion

The clonality of tumor has long been of interest to oncologists and frequently explained by "field cancerization" [32]. The concept of "field cancerization" was first proposed by Slaughter et al. in 1953 to account for multifocal tumors developed in aerodigestive tract of cigarette smokers, and defined as "increased cancer predisposition due to multiple genetic alterations or prolonged exposure to carcinogen" [33]. The internal molecular abnormalities and external environmental carcinogens contribute to malignant transformation together, thus the lateral spread of tumor occurs independently rather than metastasizes from pre-existing cancer cells [34, 35]. Since then, the concept of field cancerization has also been applied in different cancers, especially for carcinogen-exposed tissues and fields locally develop during neoplastic evolution [36, 37].

In the past few decades, the incidence of thyroid cancer was increased rapidly by 5% per year, which may be attributed to advanced image technologies detecting more tumors in the early stage [21–23]. Some researchers have demonstrated different clonality in early pre-neoplastic lesions in breast [38], intestine [39, 40] and skin [41], and whether field cancerization also exists in thyroid cancer remains unclear. In this study, we included 28 pairs of bilateral PTC in the early stage without extra-thyroidal extension, lymph node metastasis and distant metastasis, and assessed the clonal origin by XCI and BRAF mutation analysis. Although XCI assessment is the gold standard, this method is limited by the quality and quantity of DNA and cannot conduct in males or females without HUMARA polymorphism. Therefore, we combined the analysis of BRAF mutation to improve the sensitivity of our study. During transformation and progression of cancer, different genetic mutations may be accumulated in one tumor and cause genetic heterogeneity [11, 12]. Because of a high prevalence and is regarded as an early genetic event, BRAF mutation can be used as a marker of clonal analysis in thyroid cancer [14, 15].

Our results demonstrated discordant XCI or *BRAF* status in 18 pairs of bilateral tumors, providing proof for independent clonal origin in 64.3% (18/28) cases. What's more, in the other 10 bilateral PTCs, seven harbored the same *BRAF* mutation in both lesions. Considering the high frequency of *BRAF* mutation in thyroid cancer, it can't be distinguished whether this is a consequence of a single mutation spreading throughout the gland, or of unrelated mutational events in separate tumors. Similar concerns can also be applied to the interpretation of XCI status. Therefore, we conclude that at least 64.3% bilateral PTCs in the early stage are of independent clonal origin, and the proportion could be underestimated in this study.

In our previous study, we analyzed the clonal origin of bilateral PTCs with advanced stage (with lymph node metastasis, extra-thyroidal extension or tumor recurrence), and found about 80% of them shared the same clonal origin and probably arose from a single clone [19]. Furthermore, we confirmed that bilateral PTCs with lymph node

Table 4 Clonality assessment based on combination of BRAF mutation and XCI analysis

Patient ID	BRAF mutation (left/right)	XCI (left/right)	Clonality
1	MUT/MUT	NA	
2	WT/WT	NA	
3	MUT/WT	S/L	Independent
4	MUT/WT	S/S	Independent
5	WT/WT	S/Random	
6	MUT/WT	S/S	Independent
7	MUT/WT	Random/S	Independent
8	WT/MUT	S/S	Independent
9	WT/MUT	NA	Independent
10	MUT/WT	NA	Independent
11	MUT/MUT	L/S	Independent
12	MUT/WT	NA	Independent
13	MUT/WT	NA	Independent
14	MUT/MUT	S/L	Independent
15	MUT/WT	S/L	Independent
16	WT/MUT	NA	Independent
17	WT/MUT	NA	Independent
18	MUT/MUT	Random/L	
19	MUT/MUT	NA	
20	MUT/WT	NA	Independent
21	MUT/MUT	S/S	
22	WT/MUT	L/Random	Independent
23	MUT/MUT	NA	
24	WT/MUT	S/S	Independent
25	MUT/MUT	NA	
26	MUT/MUT	L/L	
27	WT/MUT	NA	Independent
28	MUT/MUT	NA	

MUT mutation, *WT* wild type,

NA non-available, the informative result is not available

metastasis is different from those without lymph node metastasis in terms of disease free survival after long-term follow-up [9]. Taken together, we consider both field cancerization and intrathyroidal metastasis may happen in bilateral PTCs with opposite outcomes. Further study will be warranted to test this hypothesis.

Clarifying the clonal origin of bilateral PTC, whether contralateral tumor is a metastatic spreading or second primary tumor, has implications for understanding carcinogenesis and evolution of thyroid cancer. The information is important to clinical practice for risk assessment, treatment, and follow-up management. Patients with bilateral, clonally unrelated tumors may have a better outcome compared with those with clonally related tumors, since metastatic spread probably have occurred in the latter group. Therefore, independent clonal origin indicates the importance of early prevention and detection, while same clonal origin may emphasize the research to metastatic mechanism, biomarkers for metastasis and systemic therapy. Our study confirms "field cancerization" in early-stage bilateral thyroid cancers, suggesting that these subtype PTCs should be treated as independent and localized tumors.

Acknowledgements This study is supported by Grants from National Natural Science Foundation of China (No. 81202141, and 81272676), the Key Project of Scientific and Technological Innovation of Zhejiang Province (No. 2015C03G2010206), National Science and Technology Major Project of the Ministry of Science and Technology of China (No. 2013ZX09506015), Medical Science and Technology Project of Zhejiang Province (No. 2011ZDA009), and Natural Science Foundation of Zhejiang Province (No. Y2110414).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval and informed consent This study was approved by the Institutional Review Board of First Affiliated Hospital, Zhejiang University School of Medicine. Informed consent has been obtained from each patient after full explanation of the purpose and nature of all procedures were used according to the Helsinki Declaration of 1975, as revised in 1983.

References

1. R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2018. CA Cancer J. Clin. **68**(1), 7–30 (2018). https://doi.org/10.3322/caac.21442
2. K.D. Miller, R.L. Siegel, C.C. Lin, A.B. Mariotto, J.L. Kramer, J. H. Rowland, K.D. Stein, R. Alteri, A. Jemal, Cancer treatment and survivorship statistics, 2016. CA Cancer J. Clin. **66**(4), 271–289 (2016). https://doi.org/10.3322/caac.21349
3. M.R. Haymart, M. Banerjee, A.K. Stewart, R.J. Koenig, J.D. Birkmeyer, J.J. Griggs, Use of radioactive iodine for thyroid cancer. JAMA **306**(7), 721–728 (2011). https://doi.org/10.1001/jama.2011.1139
4. S. Markovina, P.W. Grigsby, J.K. Schwarz, T. DeWees, J.F. Moley, B.A. Siegel, S.M. Perkins, Treatment approach, surveillance, and outcome of well-differentiated thyroid cancer in childhood and adolescence. Thyroid **24**(7), 1121–1126 (2014). https://doi.org/10.1089/thy.2013.0297
5. S.C. Pitt, R.S. Sippel, H. Chen, Contralateral papillary thyroid cancer: does size matter? Am. J. Surg. **197**(3), 342–347 (2009). https://doi.org/10.1016/j.amjsurg.2008.09.011
6. J.L. Pasieka, N.W. Thompson, M.K. McLeod, R.E. Burney, M. Macha, The incidence of bilateral well-differentiated thyroid cancer found at completion thyroidectomy. World J. Surg. **16**(4), 711–716 (1992). discussion 716-717
7. B.S. Koo, H.S. Lim, Y.C. Lim, Y.H. Yoon, Y.M. Kim, Y.H. Park, K.S. Rha, Occult contralateral carcinoma in patients with unilateral

papillary thyroid microcarcinoma. Ann. Surg. Oncol. **17**(4), 1101–1105 (2010). https://doi.org/10.1245/s10434-009-0906-6
8. W. Wang, W. Zhao, H. Wang, X. Teng, H. Wang, X. Chen, Z. Li, X. Yu, T.J. Fahey 3rd, L. Teng, Poorer prognosis and higher prevalence of BRAF (V600E) mutation in synchronous bilateral papillary thyroid carcinoma. Ann. Surg. Oncol. **19**(1), 31–36 (2012). https://doi.org/10.1245/s10434-011-2096-2
9. W. Wang, X. Su, K. He, Y. Wang, H. Wang, H. Wang, Y. Zhao, W. Zhao, R. Zarnegar, T.J. Fahey 3rd, X. Teng, L. Teng, Comparison of the clinicopathologic features and prognosis of bilateral versus unilateral multifocal papillary thyroid cancer: an updated study with more than 2000 consecutive patients. Cancer **122**(2), 198–206 (2016). https://doi.org/10.1002/cncr.29689
10. M.F. Lyon, Sex chromatin and gene action in the mammalian X-chromosome. Am. J. Hum. Genet. **14**, 135–148 (1962)
11. C. Swanton, Intratumor heterogeneity: evolution through space and time. Cancer Res. **72**(19), 4875–4882 (2012). https://doi.org/10.1158/0008-5472.CAN-12-2217
12. M. Gerlinger, A.J. Rowan, S. Horswell, M. Math, J. Larkin, D. Endesfelder, E. Gronroos, P. Martinez, N. Matthews, A. Stewart, P. Tarpey, I. Varela, B. Phillimore, S. Begum, N.Q. McDonald, A. Butler, D. Jones, K. Raine, C. Latimer, C.R. Santos, M. Nohadani, A.C. Eklund, B. Spencer-Dene, G. Clark, L. Pickering, G. Stamp, M. Gore, Z. Szallasi, J. Downward, P.A. Futreal, C. Swanton, Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N. Engl. J. Med. **366**(10), 883–892 (2012). https://doi.org/10.1056/NEJMoa1113205
13. E.C. de Bruin, N. McGranahan, R. Mitter, M. Salm, D.C. Wedge, L. Yates, M. Jamal-Hanjani, S. Shafi, N. Murugaesu, A.J. Rowan, E. Gronroos, M.A. Muhammad, S. Horswell, M. Gerlinger, I. Varela, D. Jones, J. Marshall, T. Voet, P. Van Loo, D.M. Rassl, R. C. Rintoul, S.M. Janes, S.M. Lee, M. Forster, T. Ahmad, D. Lawrence, M. Falzon, A. Capitanio, T.T. Harkins, C.C. Lee, W. Tom, E. Teefe, S.C. Chen, S. Begum, A. Rabinowitz, B. Phillimore, B. Spencer-Dene, G. Stamp, Z. Szallasi, N. Matthews, A. Stewart, P. Campbell, C. Swanton, Spatial and temporal diversity in genomic instability processes defines lung cancer evolution. Science **346**(6206), 251–256 (2014). https://doi.org/10.1126/science.1253462
14. M. Xing, Molecular pathogenesis and mechanisms of thyroid cancer. Nat. Rev. Cancer **13**(3), 184–199 (2013)
15. J.A. Knauf, X. Ma, E.P. Smith, L. Zhang, N. Mitsutake, X.H. Liao, S. Refetoff, Y.E. Nikiforov, J.A. Fagin, Targeted expression of BRAFV600E in thyroid cells of transgenic mice results in papillary thyroid cancers that undergo dedifferentiation. Cancer Res. **65**(10), 4238–4245 (2005). https://doi.org/10.1158/0008-5472.CAN-05-0047
16. Z. Lu, J. Sheng, Y. Zhang, J. Deng, Y. Li, A. Lu, J. Zhang, H. Yu, M. Zhang, Z. Xiong, H. Yan, B.H. Diplas, Y. Lu, B. Liu, Clonality analysis of multifocal papillary thyroid carcinoma by using genetic profiles. J. Pathol. **239**(1), 72–83 (2016). https://doi.org/10.1002/path.4696
17. T.M. Shattuck, W.H. Westra, P.W. Ladenson, A. Arnold, Independent clonal origins of distinct tumor foci in multifocal papillary thyroid carcinoma. N. Engl. J. Med. **352**(23), 2406–2412 (2005). https://doi.org/10.1056/NEJMoa044190
18. S.Y. Park, Y.J. Park, Y.J. Lee, H.S. Lee, S.H. Choi, G. Choe, H.C. Jang, S.H. Park, D.J. Park, B.Y. Cho, Analysis of differential BRAF(V600E) mutational status in multifocal papillary thyroid carcinoma: evidence of independent clonal origin in distinct tumor foci. Cancer **107**(8), 1831–1838 (2006). https://doi.org/10.1002/cncr.22218
19. W. Wang, H. Wang, X. Teng, H. Wang, C. Mao, R. Teng, W. Zhao, J. Cao, T.J. Fahey 3rd, L. Teng, Clonal analysis of bilateral, recurrent, and metastatic papillary thyroid carcinomas. Hum. Pathol. **41**(9), 1299–1309 (2010). https://doi.org/10.1016/j.humpath.2010.02.008
20. R.P. McCarthy, M. Wang, T.D. Jones, R.W. Strate, L. Cheng, Molecular evidence for the same clonal origin of multifocal papillary thyroid carcinomas. Clin. Cancer Res. **12**(8), 2414–2418 (2006). https://doi.org/10.1158/1078-0432.CCR-05-2818
21. T.J. O'Grady, M.A. Gates, F.P. Boscoe, Thyroid cancer incidence attributable to overdiagnosis in the United States 1981–2011. Int. J. Cancer **137**(11), 2664–2673 (2015). https://doi.org/10.1002/ijc.29634
22. C.M. Kitahara, J.A. Sosa, The changing incidence of thyroid cancer. Nat. Rev. Endocrinol. **12**(11), 646–653 (2016). https://doi.org/10.1038/nrendo.2016.110
23. L. Klotz, Cancer overdiagnosis and overtreatment. Curr. Opin. Urol. **22**(3), 203–209 (2012). https://doi.org/10.1097/MOU.0b013e32835259aa
24. S. Jegerlehner, J.L. Bulliard, D. Aujesky, N. Rodondi, S. Germann, I. Konzelmann, A. Chiolero, N.W. Group, Overdiagnosis and overtreatment of thyroid cancer: a population-based temporal trend study. PLoS ONE **12**(6), e0179387 (2017). https://doi.org/10.1371/journal.pone.0179387
25. R.P. Tufano, S.I. Noureldine, P. Angelos, Incidental thyroid nodules and thyroid cancer: considerations before determining management. JAMA Otolaryngol. Head Neck Surg. **141**(6), 566–572 (2015). https://doi.org/10.1001/jamaoto.2015.0647
26. R.A. DeLellis, Pathology and genetics of thyroid carcinoma. J. Surg. Oncol. **94**(8), 662–669 (2006). https://doi.org/10.1002/jso.20700
27. N.D. Perrier, J.D. Brierley, R.M. Tuttle, Differentiated and anaplastic thyroid carcinoma: major changes in the American Joint Committee on Cancer eighth edition cancer staging manual. CA Cancer J. Clin. **68**(1), 55–63 (2018). https://doi.org/10.3322/caac.21439
28. R.C. Allen, H.Y. Zoghbi, A.B. Moseley, H.M. Rosenblatt, J.W. Belmont, Methylation of HpaII and HhaI sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation. Am. J. Hum. Genet. **51**(6), 1229–1239 (1992)
29. W.D. Tilley, M. Marcelli, J.D. Wilson, M.J. McPhaul, Characterization and expression of a cDNA encoding the human androgen receptor. Proc. Natl Acad. Sci. USA **86**(1), 327–331 (1989)
30. S. Moniz, A.L. Catarino, A.R. Marques, B. Cavaco, L. Sobrinho, V. Leite, Clonal origin of non-medullary thyroid tumours assessed by non-random X-chromosome inactivation. Eur. J. Endocrinol. **146**(1), 27–33 (2002)
31. H. Davies, G.R. Bignell, C. Cox, P. Stephens, S. Edkins, S. Clegg, J. Teague, H. Woffendin, M.J. Garnett, W. Bottomley, N. Davis, E. Dicks, R. Ewing, Y. Floyd, K. Gray, S. Hall, R. Hawes, J. Hughes, V. Kosmidou, A. Menzies, C. Mould, A. Parker, C. Stevens, S. Watt, S. Hooper, R. Wilson, H. Jayatilake, B.A. Gusterson, C. Cooper, J. Shipley, D. Hargrave, K. Pritchard-Jones, N. Maitland, G. Chenevix-Trench, G.J. Riggins, D.D. Bigner, G. Palmieri, A. Cossu, A. Flanagan, A. Nicholson, J.W. Ho, S.Y. Leung, S.T. Yuen, B.L. Weber, H.F. Seigler, T.L. Darrow, H. Paterson, R. Marais, C.J. Marshall, R. Wooster, M.R. Stratton, P.A. Futreal, Mutations of the BRAF gene in human cancer. Nature **417**(6892), 949–954 (2002). https://doi.org/10.1038/nature00766
32. P.C. Nowell, The clonal evolution of tumor cell populations. Science **194**(4260), 23–28 (1976)
33. D.P. Slaughter, H.W. Southwick, W. Smejkal, Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. Cancer **6**(5), 963–968 (1953)
34. V.M. van Houten, C.R. Leemans, J.A. Kummer, J. Dijkstra, D.J. Kuik, M.W. van den Brekel, G.B. Snow, R.H. Brakenhoff,

Molecular diagnosis of surgical margins and local recurrence in head and neck cancer patients: a prospective study. Clin. Cancer Res. **10**(11), 3614–3620 (2004). https://doi.org/10.1158/1078-0432.CCR-03-0631
35. K. Shiga, T. Ogawa, F. Yoshida, K. Matsuura, M. Tateda, S. Saijo, T. Miyagi, T. Kobayashi, Multiple squamous cell carcinomas of the head and neck show different phenotypes of allelic loss patterns suggesting different clonal origin of carcinogenesis. Anticancer Res. **23**(5A), 3911–3915 (2003)
36. G.H. Heppner, F.R. Miller, The cellular basis of tumor progression. Int. Rev. Cytol. **177**, 1–56 (1998)
37. H. Chai, R.E. Brown, Field effect in cancer-an update. Ann. Clin. Lab. Sci. **39**(4), 331–337 (2009)
38. W.C. Kisseberth, E.P. Sandgren, Polyclonal development of mouse mammary preneoplastic nodules. Cancer Res. **64**(3), 857–863 (2004)
39. A.J. Merritt, K.A. Gould, W.F. Dove, Polyclonal structure of intestinal adenomas in ApcMin/+mice with concomitant loss of Apc+ from all tumor lineages. Proc. Natl Acad. Sci. USA **94**(25), 13927–13931 (1997)
40. M.R. Novelli, J.A. Williamson, I.P. Tomlinson, G. Elia, S.V. Hodgson, I.C. Talbot, W.F. Bodmer, N.A. Wright, Polyclonal origin of colonic adenomas in an XO/XY patient with FAP. Science **272**(5265), 1187–1190 (1996)
41. D.J. Winton, M.A. Blount, B.A. Ponder, Polyclonal origin of mouse skin papillomas. Br. J. Cancer **60**(1), 59–63 (1989)