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BRIEF REPORT

DNA profiling of tumor buds in colorectal cancer indicates that they have the same mutation profile as the tumor from which they derive

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Introduction

Tumor budding is a hallmark of aggressive disease [1]. In cancers of the colorectum, breast, esophagus, pancreas and others, the presence of tumor budding at the periphery of the tumor is associated with unfavorable histopathological features and worse patient outcome (reviewed in [2]). Best studied in colorectal cancer, tumor buds are described as detached single cells or small tumor cell clusters with features reminiscent of epithelial-mesenchymal transition (EMT). Further immunohistochemical studies have demonstrated that tumor buds frequently show nuclear expression of β-catenin and loss of E-cadherin and have an anti-apoptotic, non-proliferating, migratory and invasive phenotype (reviewed in [3]).

Tumor budding may to some degree be under genetic control. Work by Jass and others has shown that both sporadic and familial colorectal cancers with microsatellite instability (MSI) have significantly less tumor budding [4]. It has been postulated that MSI augments the immune reaction at the invasion front and consequently induces destruction of tumor buds. In support of this concept, the presence of CD8+ T-lymphocytes and CD68+ macrophages is strongly correlated with a low number of tumor buds [5]. In contrast, in cancers with APC and β-catenin mutations significantly high-degree of budding has been observed [4]. These molecular changes activate WNT signaling and translocation of β-catenin to the nucleus, where it acts as a transcription factor regulating a variety of genes involved in tumor progression.

Molecular characterization of tumor buds is necessary in order to further elucidate their role in tumor progression and metastasis. Almost all studies to this effect to date have evaluated protein profiles of budding cells by immunohistochemistry. Only very recently RNA-based studies have been published, which demonstrate that cells in tumor buds express an EMT-like phenotype [6, 7]. No studies have yet investigated the genome of tumor buds. Our aim therefore was to (1) elaborate a protocol for DNA analysis by next-generation sequencing of cells in tumor buds and (2) compare the mutation profile of budding cells to that of the main tumor.

Methods

Patients

The study started with 139 colorectal cancer patients treated at the Insel Hospital, Bern, Switzerland between 2002 and 2011. From each case, one representative tissue block was selected, sectioned and stained with an anti-pan-cytokeratin antibody (AE1/AE3) to facilitate visualization of tumor buds. Stained slides were then reviewed and ten cases with extensive tumor budding at the invasion front were selected for further study. Permission to use this material was granted by the local ethics committee.

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Immunohistochemistry

Of each case 5 μm tissues were mounted onto PEN-membrane slides, previously irradiated for 2 h by UV light. Immunohistochemistry was performed on a Bond Rx (Leica Biosystems), using the AE1/AE3 antibody (Dako, mouse monoclonal, 1:200, enzyme pre-treatment 5 min; DAB chromogen) after enzyme pre-treatment for antigen retrieval. On selected slides the VE1 antibody (Ventana, ready to use) was used to detect BRAF V600E mutant protein, in order to validate this method for BRAF mutation detection (Ventana Benchmark Ultra, Roche).

Laser capture microdissection

Laser capture microdissection was carried out using an inverted microscope (AxioObserver. Z1) with the software PALMRobo V4.2 (Zeiss) on all sections stained for pan-cytokeratin. At least two regions from the tumor center, invasion front and tumor buds were captured separately. Dissection was performed using a 10× objective to reduce tissue damage. Material was catapulted into a 500 μl tube previously impregnated with 1 μl mineral oil. DNA extraction was performed using the QIAamp DNA Micro Kit (Qiagen) [8] and fluorometric quantitation using Qubit 2.0 (Invitrogen).

Mutation analysis of KRAS and BRAF by pyrosequencing

Next, 2.5 ng DNA was mixed with 12.5 μl Pyromark PCR Master Mix, 2.5 μl Coral Con, and 2 μl of forward as well as reverse primers at a concentration of 3 μM each, in a final volume of 25 μl. PCR was performed for 40 cycles with annealing temperatures of 57 °C and 60 °C for BRAF (codon 15, V600E) and KRAS (codon 12 and 13), respectively. A commercial DNA control (EpiTect® Control DNA, Qiagen) and a non-templated control (NTC) were always included. Primer sequences were as follows: BRAF V600E forward primer 5′-TGA AGA CCT CAC AGT AAA AAT AGG-3′; BRAF reverse primer 5′-TCC AGA CAA CTG TTC AAA CTG AT-3′; KRAS 12/13 forward primer 5′-TAA GGC CTG CTG AAA ATG ACT G-3′; KRAS 12/13 reverse primer 5′-TTA GCT GTA TCG TCA AGG CAC TCT-3′. After PCR, fragment analysis was carried out using a Qiaxcel system (Qiagen). Pyrosequencing was performed using a PyroMark Q24. Sequencing primers included the following: KRAS 5′-CTT GTG GTA GTT GGA GC-3′ and BRAF 5′-TGA TTT TGG TCT AGC TAC A-3′. We considered cases to be mutated when the frequency of mutated allele was 10% or greater [9].

Next-generation sequencing

Next, all cases underwent next-generation sequencing based on Ion AmpliSeq™ Cancer Hotspot Panel v2 (CHPv2) (Thermo Fisher Scientific) amplification using an Ion Torrent PGM platform (Thermo Fisher Scientific). This panel consists of 207 primer pairs interrogating 50 oncogenes and tumor suppressor genes (catalog no. 4475346, for panel details see AmpliSeq.com). LCM material was collected as described above and DNA extraction was performed using the Arcturus® PicoPure® DNA extraction kit (Applied Biosystems) according to the manufacturer's protocol for FFPE material with minor changes. The concentration was measured with a Qubit® 2.0 fluorometer using the Qubit® dsDNA HS Assay kit (Molecular Probes). CHPv2 libraries were constructed using 1–9.9 ng of DNA from each pooled laser-captured isolate. The samples were processed using the Ion AmpliSeq™ Library Kit 2.0 according to the recommended protocols and amplified by PCR for 20 or 23 cycles. The template and enrichment steps were carried out on the Ion OneTouch™ 2 System with Ion PGM™ Template OT2 200 Kit. The samples were adjusted to a concentration of 100 pM, applied to an Ion 316™ v2 Chips, and sequenced on an Ion PGM™ instrument (all kits and instruments from Thermo Fisher Scientific). The sequencing data were analyzed using the Ion Reporter plugin for Torrent Suite™ Software (v5.0.4).

Results

Laser capture microdissection and DNA extraction for pyrosequencing

LCM was performed on all cases including the tumor center, invasion front and tumor buds. For tumor buds, only cells that clearly contained a nucleus surrounded by a rim of cytokeratin stained cytoplasm were considered (Figure 1). Captured areas ranged from 662′492 to 1′829’854 μm^2. DNA amplification and fragment analysis was possible and produced clear bands for all tissue samples, with expected results for corresponding DNA and NTC controls. Estimated concentration of DNA per sample was 0.2–0.5 ng/μl.

Pyrosequencing for detection of KRAS and BRAF mutations from tumor center, front and buds

Results are shown in Table 1. Pyrosequencing for the detection of KRAS and BRAF mutation was successfully conducted on DNA extracted from tumor buds in all cases. Interestingly, in one BRAF mutated (Case 4) and one KRAS mutated case (Case 6), tumor buds were designated as wild-type, due to the low frequency (<10%) of a mutation. As we had previously validated VE1 antibody staining for the detection of BRAF V600E mutations in colorectal cancer, we performed immunohistochemistry on Case 4 and positive staining with VE1 confirmed the mutated status of the cells in the tumor buds.

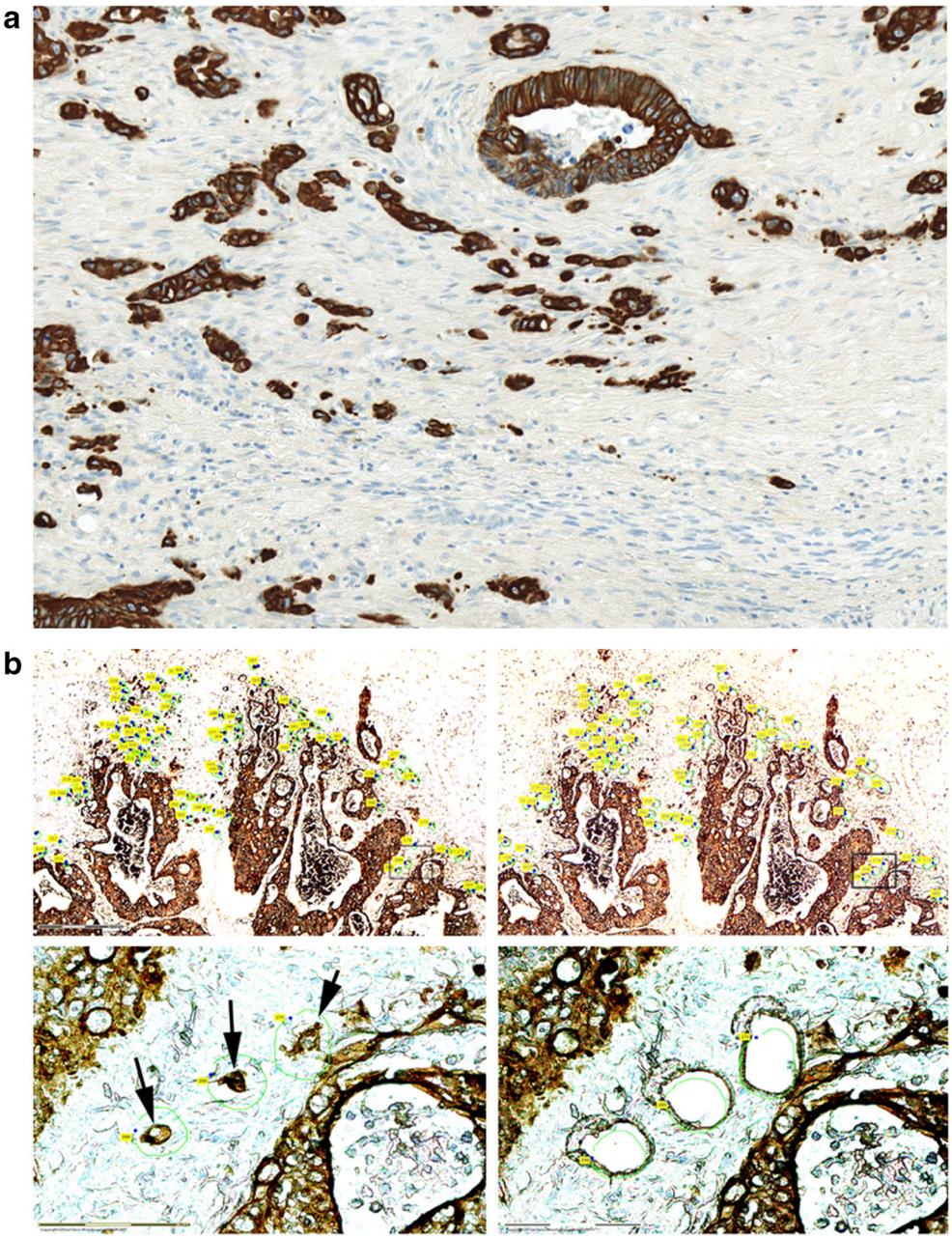

Fig. 1 a) Pan-cytokeratin (AE1/AE3) staining highlighting tumor buds at the invasion front of colorectal cancer (20× magnification). b) Laser capture microdissection showing areas before and after capture

Next-generation sequencing of tumor buds and corresponding tumor

All 10 cases underwent sequencing using the Cancer Hotspot v2 panel. To this end we again performed laser capture microdissection of pan-cytokeratin stained tissue sections and extracted DNA from the tumor bud, tumor center and the tumor invasion front samples. Details are listed in Table 2. Quality metrics underline the high quality of each sequencing run, even with the low amount of material. A library was prepared from all but one sample (center of sample 3), even with as little as 1 ng of DNA. In all cases the mutation profile of the tumor buds was identical to that of the tumor center and front. Tumor budding regions contained a lower frequency of mutated allele, which can be explained by stromal contamination during the laser capturing process.

Discussion

The aim of this study was to determine feasibility of performing molecular studies on tumor buds using pyrosequencing and next-generation sequencing. Our results show that reliable results can be obtained by isolating tumor buds by LCM after

Table 1 Summary of KRAS and BRAF heterogeneity results by pyrosequencing

Case no.	Area	BRAF status	KRAS status
1	Center	wt	wt
	Center	wt	wt
	Center	wt	wt
	Front	wt	wt
	Front	wt	wt
	Buds	wt	wt
2	Center	wt	p.G12D (52%)
	Center	wt	p.G12D (58%)
	Center	wt	p.G12D (55%)
	Front	wt	p.G12D (65%)
	Front	wt	wt (3%)
	Buds	wt	wt (4%)
3	Center	p. V600E (46%)	wt
	Front	p. V600E (41%)	wt
	Buds	p. V600E (22%)	wt
4	Center	p. V600E (22%)	wt
	Front	p. V600E (21%)	wt
	Buds	wt (6%)	wt
	Center	p. V600E (16%)	wt
	Front	p. V600E (10%)	wt
	Buds	wt (3%)	wt
5	Center	p. V600E (37%)	wt
	Front	p. V600E (17%)	wt
	Buds	p. V600E (25%)	wt
	Center	p. V600E (43%)	wt
	Front	p. V600E (34%)	wt
	Buds	p. V600E (36%)	wt
	Center	p. V600E (49%)	wt
	Front	p. V600E (32%)	wt
	Buds	p. V600E (19%)	wt
6	Center	wt	p. G13D (32%)
	Front	wt	p. G13D (19%)
	Buds	wt	p. G13D (12%)
	Center	wt	p. G13D (35%)
	Front	wt	wt (8%)
	Buds	wt	p. G13D (12%)
	Center	wt	p. G13D (41%)
	Front	wt	wt (4%)
	Buds	wt	p. (10%)
7	Center	wt	p. G13D (45%)
	Front	wt	p. G13D (45%)
	Buds	wt	p. G13D (24%)
	Center	wt	p. G13D (24%)
	Front	wt	p. G13D (13%)
	Buds	wt	p. G13D (11%)
8	Center	wt	p. G13 V*
	Front	wt	p. G13 V*
	Buds	wt	wt
	Center	wt	p. G13V*
	Front	wt	p. G13V*
	Buds	wt	wt
9	Center	wt	p. G12V (39%)
	Front	wt	wt
	Buds	wt	wt
	Center	wt	p. G12V (11%)
	Front	wt	wt
	Buds	wt	wt
10	Center	wt	p. G12S (16%)
	Front	wt	p. G12S (44%)
	Buds	wt	p. G12S (19%)
	Center	wt	p. G12S (33%)
	Front	wt	p. G12S (52%)
	Buds	wt	p. G12S (11%)

*Percentage not determined

pan-cytokeratin immunostaining. In addition, we found no differences in mutation profile between tumor buds and tumor body samples. We conclude that the process of tumor budding is not facilitated by the acquisition of additional driver mutations.

Heterogeneity of clinically relevant mutations in colorectal cancers has previously been investigated [10]. In our pyrosequencing study the 10% cutoff to call a mutation resulted in one case discordant for KRAS, when comparing tumor buds with tumor center and tumor front. In two more cases tumor buds were assigned wild-type status due to their apparent low mutation frequency (<10%), while the tumor body was BRAF or KRAS mutated. These results were confirmed in multiple independent rounds of laser capture and yet the presence of the BRAF V600E mutation was confirmed in tumor buds by VE1 immunohistochemical staining. These results show that immunohistochemical staining using the VE1 is a sensitive method for detecting low frequency V600E mutations [11].

We did find discrepancies between pyrosequencing and next-generation sequencing results in two cases (samples 8 and 9). Close investigation of case 9 showed a KRAS G12 V mutation in tissue punches taken from the center of the tumor. This finding can be explained by tumor heterogeneity. Repeated pyrosequencing analysis of case 8 did not identify a KRAS mutation in tumor buds, which can be explained by tumor heterogeneity but also by higher sensitivity of next-generation sequencing for KRAS mutation detection.

Next-generation sequencing using the cancer hotspot v2 panel was successfully performed in all 10 cases on even tiny

Table 2 Summary of NGS results taken from laser captured material of tumor center, front and tumor buds from 10 high-grade budding cases

Case no.	Area	BRAF status	KRAS status	Other mutations found
1	Center	wt	wt	NRAS p. G12A (98.6%), PIK3CA p. E545K (96.9%), FBXW7 p. S582 L (49.3%)
	Front	wt	wt	NRAS p. G12A (96.4%), PIK3CA p. E545K (96.1%), FBXW7 p. S582 L (49.3%)
	Buds	wt	wt	NRAS p. G12A (13.4%), PIK3CA p. E545K (9.1%), FBXW7 p. S582 L (8.1%)
2	Center	wt	KRAS p. G12D (58.5%)	APC p. Q1429* (45%)
	Front	wt	KRAS p. G12D (63%)	APC p. Q1429* (43.8%)
	Buds	wt	KRAS p. G12D (9.4%)	APC p. Q1429* (5.2%)
3	Front	BRAF p. V600E (37.3%)	wt	APC p. T1556fs (33.8%), PTEN p. K267fs (80.2%)
	Buds	BRAF p. V600E (6.5%)	wt	APC p. T1556fs (6.8%), PTEN p. K267fs (5.6%)
4	Center	BRAF p. V600E (49.5%)	wt	TP53 p. Q100* (97.5%)
	Front	BRAF p. V600E (47.5%)	wt	TP53 p. Q100* (91.2%)
	Buds	BRAF p. V600E (10.7%)	wt	TP53 p. Q100* (18.4%)
5	Center	BRAF p. V600E (39%)	wt	TP53 p. Q354R (29.7%)
	Front	BRAF p. V600E (26.7%)	wt	TP53 p. Q354R (23.8%)
	Buds	BRAF p. V600E (5.6%)	wt	TP53 p. Q354R (3.7%)
6	Center	wt	KRAS p. G13D (63.2%)	PIK3CA p. E542K (93.2%), TP53 p. R175H (92.9%)
	Front	wt	KRAS p. G13D (60%)	PIK3CA p. E542K (94.6%), TP53 p. R175H (95.6%)
	Buds	wt	KRAS p. G13D (6.6%)	PIK3CA p. E542K (7.9%), TP53 p. R175H (11.1%)
7	Center	wt	KRAS p. G13D (65.8%)	
	Front	wt	KRAS p. G13D (60.1%)	
	Buds	wt	KRAS p. G13D (8.3%)	
8	Center	wt	KRAS p. G13 V (65%)	
	Front	wt	KRAS p. G13 V (62.1%)	
	Buds	wt	KRAS p. G13 V (16.3%)	
9	Tumor	wt	KRAS p. G12 V (48.5%)	PIK3CA p. E542K (48.8%), FBXW7 p. R505C (6.2%), APC p. S1356* (46.6%)
	Buds	wt	KRAS p. G12 V (2.7%)	PIK3CA p. E542K (2.5%), APC p. S1356* (3.4%)
10	Center	wt	KRAS p. G12S (22.1%)	PIK3CA p. V344G (47.7%)
	Front	wt	KRAS p. G12S (48.9%)	PIK3CA p. V344G (60.4%)
	Buds	wt	KRAS p. G12S (10.1%)	PIK3CA p. V344G (7.5%)

amounts of laser captured and immunostained material. The mutation profile of tumor buds was identical to that of the main tumor; no additional mutations were identified in cells in tumor buds. These results demonstrate that tumor buds are likely not derived from a more aggressive tumor clone and that the process of budding is not associated with changes at DNA level.

De Smedt et al. recently published results of RNA analysis of 296 genes on laser captured tumor buds [6]. They compared expression profiles of tumor buds and the main tumor and related these to the consensus molecular subtypes (CMS) [12]. They found expression profiles consistent with a switch from a more epithelial CMS2 in the tumor center to a more mesenchymal CMS4 subtype in the tumor buds, which emphasizes the link between tumor buds and EMT. Similar results were found by Jensen and colleagues who performed expression profiling of tumor budding cells in oral squamous cell carcinoma [7]. They found a gene expression signature in budding cells consistent with an EMT profile and activation of TGFβ signaling. Tumor buds consist of epithelial cells and are therefore pan-cytokeratin positive [1]. We hypothesize that pan-cytokeratin positive buds are composed of a subset of cells that are entering into EMT, i.e. representing partial EMT.

Our work shows that molecular analysis can be successfully performed on tumor buds, despite the low amount of DNA that is retrieved. We show that tumor buds have the same mutation profile as the tumor from which they derive, within the limits of the set of driver mutations we analyzed. These results support the concept that the process of tumor budding is not due to the acquisition of new mutations characterizing a more aggressive clone.

Compliance with ethical standards Permission to use the material for this study was given by the cantonal ethics commission (KEK 200/2014).

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Conflict of interest None of the authors have any conflicts of interest.

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