The majority of β-catenin mutations in colorectal cancer is homozygous

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Abstract

Background: β-catenin activation plays a crucial role for tumourigenesis in the large intestine but except for Lynch syndrome (LS) associated cancers stabilizing mutations of β-catenin gene (CTNNB1) are rare in colorectal cancer (CRC). Previous animal studies provide an explanation for this observation. They showed that CTNNB1 mutations induced transformation in the colon only when CTNNB1 was homozygously mutated or when membranous β-catenin binding was hampered by E-cadherin haploinsufficiency. We were interested, if these mechanisms are also found in human CTNNB1 mutated CRCs.

Results: Among 869 CRCs stabilizing CTNNB1 mutations were found in 27 cases. Homo- or hemizygous CTNNB1 mutations were detected in 74% of CTNNB1 mutated CRCs (13 microsatellite instable (MSI-H), 7 microsatellite stable (MSS)) but only in 3% (1/33) of extracolonic CTNNB1 mutated cancers. In contrast to MSS CRC, CTNNB1 mutations at codon 41 or 45 were highly selected in MSI-H CRC. Of the examined three CRC cell lines, β-catenin and E-cadherin expression was similar in cell lines without or with heterozygous CTNNB1 mutations (DLD1 and HCT116), while a reduced E-cadherin expression combined with cytoplasmic accumulation of β-catenin was found in a cell line with homozygous CTNNB1 mutation (LS180). Reduced expression of E-cadherin in human MSI-H CRC tissue was identified in 60% of investigated cancers, but no association with the CTNNB1 mutational status was found.

Conclusions: In conclusion, this study shows that in contrast to extracolonic cancers stabilizing CTNNB1 mutations in CRC are commonly homo- or hemizygous indicating a higher threshold of β-catenin stabilization to be required for transformation in the colon as compared to extracolonic sites. Moreover, we found different mutational hotspots in CTNNB1 for MSI-H and MSS CRCs suggesting a selection of different effects on β-catenin stabilization according to the molecular pathway of tumourigenesis. Reduced E-cadherin expression in CRC may further contribute to higher levels of transcriptionally active β-catenin, but it is not directly linked to the CTNNB1 mutational status.

Keywords: Colorectal cancer (CRC), β-catenin (CTNNB1), E-cadherin
Background
Molecular biological studies over the last 4 decades have unraveled the multidimensional role of β-catenin (encoded by the CTNNB1 gene) in embryological development and carcinogenesis [1]. Physiologically, β-catenin acts as a regulator of gene expression in the WNT-signaling pathway and as a crucial component of cell-cell-adhesion. In short, activating WNT signaling results in cytoplasmatic accumulation of free β-catenin and its subsequent nuclear translocation, where it functions together with other coactivators as an active transcriptional complex for the expression of WNT target genes [2, 3]. On the other hand, β-catenin forms a complex with E-cadherin, which mediates cell-cell-adhesion, thereby maintaining epithelial tissue integrity [4, 5].

Frequent heterozygous mutations in hotspot region of exon 3 in CTNNB1 have been described for different tumour entities such as hepatocellular carcinoma [6], solid pseudopapillary tumour [7] and desmoid fibromatosis [8]. In contrast, CTNNB1 mutations are rare in CRC. Here, the activation of the WNT pathway appears to be mostly driven by a bi-allelic alteration of the APC gene resulting in decreased β-catenin degradation [9]. However, alterations in CTNNB1 itself represent a well characterized alternative to the canonical APC driven pathway activation [10]. Deletions and mutations in the hotspot of exon 3 lead to a loss of serine/threonine phosphorylation sites impeding the degradation of β-catenin by the destruction complex, thereby increasing cytoplasmic β-catenin protein levels [11]. Moreover, in CRCs, APC and CTNNB1 mutations are almost always mutually exclusive and associated with different genetic signatures [12]. While APC mutations are found more frequently in microsatellite stable (MSS) CRCs, CTNNB1 mutations are commonly associated with microsatellite instability (MSI-H) particularly in cancers from Lynch syndrome patients [13, 14].

Besides the activation of the WNT pathway, alterations of CTNNB1 may influence E-cadherin-mediated cell-cell-adhesion. These aberrations are known to be associated with reduced protein levels of membranous E-cadherin and the expression of markers of epithelial-mesenchymal transition (EMT), thereby possibly facilitating metastatic spread of malignant cells [15, 16]. Moreover, Huels et al. (17) have linked these complex interactions between β-catenin and E-cadherin to the different tumorigenic potential of CTNNB1 mutations in the colon compared to other anatomic locations. In their animal model study they show that heterozygous CTNNB1 mutations are drivers of tumourigenesis in the small intestine while in the large bowel homozygous mutations of CTNNB1 are necessary for tumour development.

In order to study the characteristics of CTNNB1 mutations in CRC and their association with altered E-cadherin expression, we evaluated 869 CRCs for CTNNB1 mutations and analysed the expression of E-cadherin and β-catenin in three cell lines with CTNNB1 wildtype (DLD1), heterozygous (HCT 116), and homozygous mutations (LS 180).

Methods
CRC cohorts
Two CRC cohorts were analysed
Cohort 1 consisted of 839 CRCs panel sequenced for diagnostic purposes from January 2015 to December 2017 in the Department of Pathology, Charité University Medicine Berlin. Samples were designated for companion (a theranostic testing of the K-RAS/N-RAS status that indicate a patient’s response to an EGFR-inhibitor) diagnostics (826 CRC) or for BRAF analysis to distinguish sporadic BRAF mutated from potentially Lynch Syndrome associated MLH1 deficient MSI-H CRCs without BRAF mutation (13 MLH1 deficient MSI-H CRCs). MSI status was known for 221 of the CRCs. Thirty one CRCs were MSI-H and 190 CRCs were MSS.

Cohort 2 included CRCs from a scientific study on somatic mutations in 30 CRCs associated with Lynch syndrome (LS). LS is caused by germline mutations in one of the DNA mismatch repair genes MLH1, MSH2, MSH6, or PMS2. In the present cohort LS associated CRCs were deficient for the DNA mismatch repair proteins MLH1, MSH2, MSH6, or PMS2 in 13, 11, 4, and 2 LS CRCs, respectively.

For 21 cases of cohort 2 data on the presence or absence of CTNNB1 mutations has been reported previously [17].

The study was approved by the ethics committees of Berlin and Heidelberg (Application Berlin EA4/003/19 and EA1/413/16 and Heidelberg S-583/2016).

Extracolonic cancer cohort
To compare the findings of homozygous CTNNB1 in CRCs with those in extracolonic cancers we analysed panel sequenced lung cancers and melanomas. The Lung cancer cohort consisted of 23 non-small cell lung cancer analysed for EGFR mutations and the melanoma cohort of 10 metastasized melanomas analysed for BRAF mutations. Cancers were panel sequenced for therapy predictive purposes from January 2015 to December 2017 in the Department of Pathology, Charité University Medicine Berlin.

Scoring of mono- and biallelic CTNNB1 mutations in CRC
Biallelic mutations are defined by mutations in both, the maternal and paternal allele copy. Both copies may harbour the same mutation (homozygous), one copy may be mutated while the second copy is lost.
(hemizygous), or both copies may be affected by different mutations (compound heterozygous).

While detection of compound heterozygous mutations is straightforward, the interpretation of homo- or hemizygous mutations in cancer tissue is hampered by admixed non-neoplastic cells, which dilute tumorous DNA and thus lower the proportion of cancerous mutations. In routine practice, DNA isolated from cancers is “contaminated” by 20–70% of DNA from non-neoplastic cells. To score CTNNB1 mutations for mono- or biallelic mutations we followed expected mutational allele frequencies (AF) for tumor specific somatic mutations in samples with varying tumor cell purity as described previously [18].

In brief, we calculated the expected AFs of heterozygous and homo- or hemizygous mutations for different gene copy numbers and tumor cell purities. We defined mutations in genes typically mutated heterozygously in CRC as heterozygous reference mutations. For this purpose mutated AFs of oncogenes (KRAS, PIK3CA) where used as reference mutations for comparison with CTNNB1. In cases without mutations in oncogenes, the AF of mutated FBXW7 was evaluated. Despite being considered a tumor suppressor, FBXW7 mutations are almost always heterozygous in CRC [19].

We reasoned that irrespective of the tumor cell purity the AF of a homozygous mutation is twice as high as the AF of a heterozygous mutation. Accordingly, a CTNNB1 mutation was scored as biallelic/homozygous when its AF was twice as high as the AF of a reference gene (criterion for homozygosity). Heterozygous mutations were scored when the AF of the CTNNB1 was as high as that of the reference mutation.

In CTNNB1 mutated CRCs without reference mutations, we concluded that AFs ≥50% cannot result from heterozygous mutations. In these cases we considered homo- or hemizygous mutations. Biallelic mutations with retention of a wild type copy (mut/wt/wt for DLD1, del45S/wt/wt) were considered unlikely due to the demonstration of wild type loss by RNA sequencing, the high tumour cell purity required to exceed an AF of 50%.

We therefore scored CTNNB1 mutations in CRCs without reference mutations as biallelic/homo- or hemizygous when the AF was ≥50% (criterion for homo-or hemizygous mutation) and as heterozygous when the AF of the CTNNB1 mutation was < 50%.

**Statistical analysis**

Statistical analysis was performed using RStudio version 1.1.463 based on the statistical language R version 3.5.1. The chi square test was used to calculate significance for categorical variables. The level of significance was set at $p < 0.05$.

**Next generation sequencing**

After microscopic identification of areas with highest tumor cell concentration, DNA was isolated using a commercial DNA Extraction Kit (DNeasy; Qiagen) following the manufacturer’s protocol.

In cohort 1, multiplex PCR-based amplicon high-throughput sequencing was performed using the Ion Torrent system (ThermoFisher Scientific) according to the manufacturer’s recommendations. Briefly, 10 ng of genomic DNA was used for library construction with the Ion AmpliSeq Colon and Lung Cancer Panel v2 and the Ion AmpliSeq Library Kit 2.0 (ThermoFisher). Library quantification was carried out on the StepOne Plus Real-Time PCR System employing the Ion Library Taq-Man Quantitation Kit (both ThermoFisher). Data analysis was performed using the Sequence Pilot software (JSI medical systems GmbH). Mean sequencing depth for CTNNB1 exon 3 and commonly heterozygous CRC mutations (KRAS, PIK3CA, FBXW7) was 1816 (range 569–4241) and 1308 (range 482–3671), respectively.

CRCs from cohort 2 were analysed with more extended customized panels as previously reported [17]. Mean sequencing depth for CTNNB1 exon 3 and commonly heterozygous CRC mutations (KRAS, PIK3CA, FBXW7) was 6144 (range 1891–11,334) and 3473 (range 1342–9467), respectively.

**RNA sequencing of CTNNB1**

To confirm the homozygosity of CTNNB1 mutations, RNA was isolated from slides obtained from tumor tissue blocks using the RNeasy FFPE-Kit (Qiagen, Germany) according to the manufacturer’s protocol.

Primers spanning from exon 1 to exon 4 of the CTNNB1 mRNA were used.

RNA CTNNB1 forward 5′-GTC GAG GAC GGT CGG ACT-3′.

RNA CTNNB1 reverse 5′-CAG GAC TTG GGA GGT ATC CA-3′.

For single step generation of cDNA and PCR amplification we used the Invitrogen one step RT PCR KIT with platinum Taq (Fisher Scientific) according to the manufacturer’s protocol. PCR consisted of a single reverse transcription step at 55°C for 30 min, followed by denaturation at 94°C for 2 min and 40 cycles consisting of a denaturation (94°C for 15 s), annealing (60°C for 30 s), and an extension step (68°C for 1 min). PCR products were analysed by Sanger sequencing.

**Cell culture**

Colorectal cancer cell lines DLD1 were purchased from Horizon, HCT116 and LS180 from ATCC. The reported mutational status of CTNNB1 (wt/wt for DLD1, delH5S/
was confirmed by DNA Sanger sequencing. DLD1, HCT116, and LS180 cells were cultured in RPMI medium, DMEM supplemented with 2 mM Ultra-glutamine (Lonzar), or EMEM, respectively, each containing 10% FCS and 100 U/ml penicillin-streptomycin (Biochrom).

**Immunostaining of cell cultures**

After reaching 70–80% confluence, cells were harvested for immunofluorescence staining. Therefore, cells were washed with PBS twice and fixed with 4% formaldehyde for 30 min. Subsequently, cells were scratched from the plate using a spatula, and transferred in 15 ml Falcon tubes. Scratched cells in PBS were centrifuged for 2 min at 1200 rpm. The pellet was resuspended in histogel (Thermo Scientific) at 70 °C, transferred into a histo cassette, embedded into paraffin, and cut in 3–4 μm sections. For deparaffinization of paraffin sections, slides were incubated in xylene for 5 min three times followed by rehydration in descending dilutions of ethanol in water (100, 90, 80, 70, 0%) for 5 min each at room temperature. For antigen unmasking, slides were incubated in citrate antigen retrieval buffer (82 mM trisodium citrate dihydrate, 18 mM citric acid monohydrate, adjust to pH 6.0) for 20 min at 100 °C, and unspecific binding sites were blocked by incubation with Peroxidase-Blocking Solution (Dako) for 30 min at room temperature. Slides were incubated with primary antibodies (Rabbit anti-β-catenin (#9562 Cell Signaling), Mouse anti-E-cadherin (#14472 Cell Signaling), Rabbit anti-β-tubulin (#2146 Cell Signaling), all 1:1000) was carried out overnight at 4 °C, incubation with secondary antibodies (horse anti-mouse HRP-linked IgG; 7076S or goat anti-rabbit HRP-linked IgG 7074S, both Cell Signaling), was carried out for 1 h at room temperature. Protein bands were detected using the ECL Western Blot Detection Reagent (Amersham) according to the manufacturer’s protocol. All incubation steps were followed by washing the nitrocellulose membranes with TBST three times. Antibodies were diluted in 5% milk powder in TBST.

**Immunohistochemistry of human CRC samples**

Formalin-fixed and paraffin-embedded tissue samples of CRC were cut into 4 μm sections. A BenchMark XT immunostainer (Ventana Medical Systems, Tucson, AZ) was used for subsequent immunohistochemical staining. For antigen retrieval, sections were incubated in CCI mild buffer (Ventana Medical Systems, Tucson, AZ) for 30 min at 100 °C. Sections were stained with anti-E-cadherin antibody (clone 4A2C7, 1:50, Zytomed) for 12 min each. The stains were evaluated using an Olympus BC50 microscope and Olympus PLN 4X/0.1 Plan Achromat Objectives (Olympus Europe Holding GmbH, Hamburg, Germany). Histological images were acquired with the digital camera moticam 3.0 mp (Motic Instruments Inc., California, USA) and Motic Images Plus software (Motic instruments Inc.).
Results
CTNNB1 mutations in CRC
Among 839 CRCs from the diagnostic cohort we detected 16 cases with CTNNB1 mutations (1.9%), which comprised 7 MSI-H and 9 MSS tumors. The 7 CTNNB1 mutated MSI-H CRCs included 3 tumors analysed for LS diagnostics and one metastasized tumor from an LS patient analyzed for companion diagnostics. LS status was unknown in the remaining 3 CTNNB1 mutated MSI-H CRC sequenced for therapy prediction. None of the CTNNB1 mutated MSI-H CRCs carried a BRAF mutation.

Among 30 LS CRCs from the scientific cohort we detected 11 CTNNB1 mutations (37%). Nine CRCs were MLH1 deficient and one tumor each was MSH2 and PMS2 deficient.

Among the total of 27 CTNNB1 mutated CRCs of both cohorts, CTNNB1 mutations in 13 CRCs (2 MSS, 11 MSI-H) were scored as biallelic/homozygous. Values for CTNNB1 AFs relative to the AFs of reference mutations were highly concordant with expected values and ranged between 1.9 and 2.1. Only CTNNB1 mutations in two CRCs displayed relative values of 2.4 and 3.1. Given a near perfect match of the relative AFs with that calculated for 3 and 4 mutated allele copies in the absence of wild type alleles, respectively, these tumours were also scored as homozygous.

In 10 out of 13 CRCs with homozygous CTNNB1 mutations the AFs of mutated CTNNB1 exceeded 50%.

CTNNB1 mutations in 7 CRCs without a reference mutation had AFs >60% (range 62–78%) and were scored as biallelic/ homo- or hemizygous.

CTNNB1 mutations in 7 CRCs were scored monoallelic/heterozygous.

The results are summarized in Table 1 and the supplementary Table 1.

CTNNB1 RNA sequencing
The homozygous nature of CTNNB1 mutations was exemplarily confirmed by RNA sequencing of CTNNB1 in four cases, in which DNA sequencing revealed CTNNB1 mutations with AFs ranging from 50 to 63% (Fig. 1). In all four cases, wild type CTNNB1 RNA was absent or minimally present. Remnants of wild type CTNNB1 were considered to result from expression of CTNNB1 by stromal cells.

Zygosity of CTNNB1 mutations in the extracolonic cancer cohorts
None of the 23 lung cancers with CTNNB1 mutations fulfilled the criterion for biallelic/ homozygous mutation and one tumor displayed a CTNNB1 AF of 57% and thus was scored as biallelic/homo- or hemizygous. The remaining 22 lung cancers and all 10 CTNNB1 mutated melanomas were scored as monoallelic/heterozygous.

Using the chi square test we calculated the significance of the frequency of homo/hemizygous mutations in CRC vs. lung/melanoma cancers resulting in a p-value <0,001.

Comparison of CTNNB1 mutational hotspots in MSI-H and MSS cancers
In accordance with previous reports, we found the frequency of CTNNB1 mutations to be higher in MSI-H CRC than in MSS CRC and our data indicate that among the MSI-H CRC CTNNB1 mutations are mainly contributed by LS-associated tumors. We also discovered that not only the frequency, but also the type of CTNNB1 mutations differed between MSI-H and MSS CRCs. All MSI-H cancers displayed CTNNB1 mutations affecting either residue T41 or S45. The majority of alterations were missense mutations while two cases displayed 3 bp deletions at codon 45 (S45del) and one tumor harbored a 12 bp deletion (delS45-G48).

In MSS cancers, mutations were more evenly distributed among mutational hotspots. We detected in-frame deletions in 4 cases overlapping at codons 25–37, and 5 missense mutations at codon 34, 37, 41 (2), and 45 (Table 1, Fig. 2).

Using the chi square test we calculated significance of the hotspot mutations in MSI vs. MSS CRCs resulting in a p-value <0,030.

β-Catenin and E-cadherin expression in cell lines
In order to gain an insight into the association of CTNNB1 mutations with E-cadherin expression, we

| Cohort     | Diagnostic | Diagn. MSS | Diagn. MSI-H | Scientific |
|------------|------------|------------|--------------|------------|
| CTNNB1 mutated | 16/839 (1.9%) | 9 | 7 | 11/30 (37%) |
| biallelic   | 12/16      | 7/9        | 5/7          | 8/11       |
| homozygous  | 6          | 2          | 4            | 7          |
| homo- or hemizygous | 6 | 5          | 1            | 1          |
| heterozygous| 4          | 2          | 2            | 3          |
studied β-catenin and E-cadherin levels in different MSI-H CRC cell lines by western blot analysis and immunofluorescence microscopy. Interestingly, we observed that β-catenin protein levels were similar in all three cell lines analyzed (DLD1: wildtype/wildtype, HCT116: wildtype/S45del, LS180: S45F/S45F). However, E-cadherin protein levels were significantly lower in LS180 than in DLD1 and HCT116 cells. This difference in E-cadherin protein levels could also be seen by immunohistochemistry. We further observed that LS180 cells displayed an incomplete, somewhat patchy E-cadherin distribution along the cell membrane while DLD1 and HCT116 showed a complete and distinct membrane staining of E-cadherin. Immunofluorescence with co-staining of β-catenin and E-cadherin confirmed the irregular distribution pattern E-cadherin in LS180 cells seen by immunohistochemistry. It further showed that this phenotype is associated with a diffuse cytoplasmic localization of β-catenin. In contrast, in DLD1 and HCT116 cells, β-catenin and E-cadherin are mainly colocalized along the cell membrane (Fig. 3) while only single cells in HCT116 displayed cytoplasmic and nuclear staining of β-catenin.

**E-cadherin expression in human CRCs with and without CTNNB1 mutation**

In vitro, we found that homozygous CTNNB1 mutation at residue S45 might perturb E-cadherin-mediated cell-cell adhesion. Hence, we questioned if CTNNB1 mutations found in CRCs also have an impact on E-cadherin expression.
Therefore, we analyzed the E-cadherin expression in 17 MSI-H CRCs with known CTNNB1 mutational status (10 homo- or hemizygous, 2 heterozygous, 5 wildtype). Strong reduction of E- Cadherin was found in 11 cancers while 6 CRCs displayed an expression similar to that in surrounding epithelium (Fig. 3). Aberrant expression of E-cadherin, however, was neither exclusive to CTNNB1-mutated tumours nor did all CTNNB1 mutated CRCs display reduced E-cadherin expression. Three out of 5 CRCs (60%) without CTNNB1 mutations and 8 of 12 CRCs (66%) with CTNNB1 mutations showed a reduced concentration of membranous E-cadherin while normal E-cadherin expression was found in 2 CRCs without and 4 CRCs with CTNNB1 mutations.

**Discussion**

This is the first study systematically analysing CRCs for biallelic mutations in the β-catenin gene. Using absolute and relative allele frequencies of CTNNB1 mutations to score zygosity we detected 71% of CTNNB1 mutated CRCs (20/28) harbouring biallelic mutations mainly due to mutational homozygosity.

One limitation of a computational method in scoring zygosity is that not all factors influencing mutated allele frequency are considered. For example copy number gains might have affected the mutated CTNNB1 allele and thus elevated the mutated allele frequency. Moreover, we cannot exclude that tumour heterogeneity lowered the mutated allele frequencies of the reference mutations, particularly in PIK3CA, and thus could have resulted in a false interpretation of CTNNB1 mutational homozygosity.

To exclude a significant impact of these limitations we therefore compared the results in CRC to those in extracolonic tumour types, which should be affected equally by a systematic methodical limitation. In these extracolonic cancers, however, the computational method did not reveal homozygous CTNNB1 mutations. Among 33 extracolonic CTNNB1 mutated cancers we detected only a single lung cancer fulfilling the criterion for a hemizygous mutation (4%). These data clearly delineate the CTNNB1 mutational zygosity findings in CRCs from those in cancers outside the colon (74% versus 4%; p < 0.001).

While CTNNB1 alterations have been investigated extensively in human cancers, only a few studies have drawn attention to biallelic mutations. Reports of homozygous CTNNB1 mutations are found for parathyroid adenoma [20] and for two colorectal adenomas [21, 22]. Moreover, Rebouissou et al. recently reported homo- and hemizygous mutations of CTNNB1 in hepatocellular carcinoma. In that study, double hit mutations were restricted to codon 45 while mutations at β-TrCP binding site (codons 32–37) and at codon 41 were heterozygous [23]. Using functional assays, the authors found that mutations at codon 45 only led to a weak activation of β-catenin compared to mutations at codon 41 and at the β-TrCP binding site, which resulted in moderate and strong activation, respectively. It was concluded that
homozygous mutations at S45 are necessary to confer an oncogenic effect strong enough to induce a malignant tumour.

A similar explanation may also apply to CTNNB1-mutated CRC. In previous animal studies, Huels et al. found that a single CTNNB1 mutation drives tumorigenesis in the small bowel, but is insufficient to induce transformation in the colon [24]. The discrepant effect in the small and the large bowel was explained by a higher expression of E-cadherin which binds excessive β-catenin to the membrane and thus compensates for the oncogenic effect of single hit CTNNB1 mutations in the colon. The tumour suppressive effect of E-cadherin, however, is overcome in the presence of homozygous CTNNB1 mutations indicating that threshold exceedance of mutated β-catenin levels also plays a role in colorectal neoplasia.

Apart from doubling the number of mutated alleles in tumour cells, homozygous CTNNB1 mutations in colorectal cancer also result in a loss of wild type β-catenin, a finding usually associated with inactivation of a tumour suppressor. The presence of CTNNB1 mutational homozygosity in cancers at a specific site, like the colon, may suggest that oncogenic activation acts in concert with a loss of tissue-dependent tumour suppression. One such β-catenin function results from its engagement in the formation of adherens junctions by mediating the mechanical link of E-cadherin to the actin cytoskeleton. Two previous analyses on HCT116, a cell line which carries a heterozygous CTNNB1 mutation at

**Fig. 3** E-cadherin and β-catenin expression in colorectal cancer cell lines DLD1 (CTNNB1 wt/wt), HCT116 (CTNNB1 S45del/wt), and LS180 (CTNNB1 S45F/S45F). a Strongly reduced E-cadherin expression in LS180 as compared to DLD1 and HCT116. b E-cadherin and β-catenin immunofluorescence staining showing reduced levels and aberrant membranous localization of E-cadherin only for LS180 cells Distinct membranous colocalization of E-cadherin and β-catenin in DLD1 and HCT116 (long white arrows) and dot-like discontinuous expression of E-cadherin and β-catenin in LS180 (short arrows). (original magnification x400). c Immunohistochemistry of E-cadherin in tumour samples from CRC patients. Upper image. Tumour specific (T) reduction of E-cadherin expression in comparison to non neoplastic crypts (N) in a CTNNB1 wildtype CRC. Lower image: Normal tumor specific E-cadherin expression in a CTNNB1 mutated CRC (original magnification x 200).
codon 45, reported that knockdown of the wild type copy resulted in the loss of E-cadherin-mediated adhesion function [25, 26]. In accordance with these findings, we detected reduced E-cadherin levels and aberrant membranous distribution exclusively in LS180 cells, the only CRC cell line we investigated, which harbours a homozygous CTNNB1 mutation. Further E-cadherin analysis in human cancers, however, drew a more complex picture. Reduced E-cadherin expression was neither exclusive to CRCs with homozygous CTNNB1 mutations, nor did all CRCs with homozygous mutations exhibit an aberrant E-cadherin expression. Thus, our data do not show an exclusive link between CTNNB1 mutations and reduced E-cadherin expression in human CRC tissue.

The reason for the divergent results in cell culture and human tissue analyses is not clear. It is possible, that the reduction of E-cadherin expression promotes tumour development by enhancing β-catenin nuclear activity, yet is caused by mechanisms different from CTNNB1 homozygous mutations in both, CRC cell line LS180 and CRC tissue. Beside transcriptional downregulation of E-cadherin by a factor acting in concert with β-catenin stabilization one such mechanism could be the mutational inactivation of δ-catenin (p120), a protein that stabilizes E-Cadherin at the cell membrane. Interestingly, biallelic mutations of the δ-catenin gene, CTNND1, have recently been proposed to cause lack of E-cadherin expression in the CRC cell line SW48 [27].

Finally, the present study confirms the association of CTNNB1 mutations with LS associated CRC and further detected different hotspot loci according to the microsatellite status. All MSI-H CRCs displayed mutations affecting either codon 41 or 45 while mutations in MSS CRCs were more evenly distributed and included mutations within the β-TRcP binding site at residues 32–37 (Fig. 2). These findings correspond well to results of previous CRC sequencing studies reported by Hampel et al. and Yaeger et al. [28, 29]. In both studies, codons 41 and 45 of CTNNB1 were found as mutational hotspots in MSI-H cancers while in MSS cancers larger deletions including the β-TRcP binding site dominated.

The reason for the selection of codon 41 or 45 mutations in MSI-H cancers is as unclear as the timing of CTNNB1 first and second hit mutations in the colorectal adenoma to carcinoma sequence. If microsatellite instability precedes CTNNB1 mutations as previously proposed by Ahadova et al. [30], the selection may be due to mutational events favoured by defective DNA-mismatch repair as already shown for mutations in APC [31]. On the other hand, several molecular studies on sporadic colorectal adenomas have revealed CTNNB1 mutations preferentially at codons 41 and 45 at a significantly higher frequency than in CRC [31, 32]. Therefore it is possible that hetero- and homozygously CTNNB1 mutated adenomas are at low risk to transform into cancer unless they acquire DNA mismatch repair deficiency as just the right mechanism to switch to malignancy.

Conclusions
In conclusion, we herein show that CTNNB1 mutations in CRC are frequently homozygous. Moreover, we found that the known association of CTNNB1 mutations with LS-CRCs is restricted to mutations at codons 41 and 45, while in MSS CRCs mutations are more evenly distributed and commonly consist of larger in-frame deletions. This selection of specific mutations indicates a “just right” level of β-catenin stabilization to be essential for MSS- and MSI-H driven carcinogenesis. Consequently, aberrant activation of the Wnt signalling pathway might play different roles in the development and progression of MSS- and MSI-H-CRC. These findings are especially important in the context of evolving new target therapies of β-catenin which as yet, however, have not been incorporated into clinical practice [33, 34].

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12885-020-07537-2.

Additional file 1: Supplementary Table S1.
Additional file 2.

Abbreviations
AF: Allele frequency; CRC: Colorectal cancer; CTNNB1: β-catenin;
EMT: Epithelial-mesenchymal transition; IHC: Immunohistochemistry;
LS: Lynch syndrome; MSI: Microsatellite instability/ microsatellite-unstable;
MSS: Microsatellite stability/ microsatellite stable

Acknowledgements
Not applicable.

Authors’ contributions
AA: Design of the study, data collection, writing of the manuscript; MT: Data collection, writing of the manuscript; CS: Intellectual input, critical discussion of the manuscript; AHA: Data collection, critical discussion of the manuscript; EV: Intellectual input, critical discussion of the manuscript; SM: Data collection, Intellectual input; HD: critical discussion of the manuscript; MM: Data collection; PB: data analysis, writing of the manuscript; MK: Design of the study, data analysis, writing of the manuscript, HB: Design of the study, data analysis, writing of the manuscript. The authors have read and approved the manuscript.

Funding
Grant support: This study was supported by Wilhelm Sander Foundation (2016-0261). Open Access funding enabled and organized by Projekt DEAL.

Availability of data and materials
The authors confirm that the data supporting the findings of this study are available within the article its supplementary materials. However, the datasets used and/or analysed during the current study are not publicly available due to data protection laws but are available from the corresponding author on reasonable request.
Ethics approval and consent to participate
The study was approved by the ethics committees of Berlin and Heidelberg (Application Berlin EA/003/19 and EA/1413/16 and Heidelberg S-583/2016). All participants signed an informed consent.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 29 May 2020 Accepted: 16 October 2020

Published online: 28 October 2020

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