Introduction

Colorectal cancer (CRC) is a leading cause of cancer death worldwide. In 2011, CRC accounted for an estimated nine percent of new cancer cases, as well as nine percent of cancer deaths in the US [1]. For the treatment of advanced CRC (stage IV), two chemotherapeutic options are available: 5-Fluorouracil (5-FU, capecitabine) in combination with irinotecan (FOLFIRI) or oxaliplatin (FOLFOX) plus biological agents. Several studies report similar response rates between the two regimens in first line treatment of advanced disease [2–4], with a single study reporting a significantly higher response rate with FOLFOX [5]. Interestingly, one of these studies reported a second line 6% objective response to FOLFIRI treatment following failed FOLFOX and a
21% objective response to second line FOLFOX treatment following failed FOLFIRI, indicative of non-complete cross resistance between irinotecan and oxaliplatin [4]. This finding raises the question of whether a subset of patients that received FOLFOX as first line treatment would have benefited from FOLFIRI as first line treatment, or vice versa. We therefore consider that efforts directed at the discovery of a predictive biomarker profile for FOLFOX/FOLFIRI treatment outcome are warranted.

Irinotecan, a pro-drug of SN-38, functions by inhibiting the enzyme topoisomerase I (Top1) [6]. Top1 plays an essential role in alleviating the topological stresses that arise during DNA replication and transcription by nicking, relaxing and re-ligating the double-stranded DNA structure. SN-38 binds Top1 and stabilizes the intermediate DNA-Top1 complexes. Subsequent re-ligation is inhibited, which ultimately results in cell death due to DNA damage during DNA replication or transcription [6,7]. Top1 has due to its role as a target for SN-38 been proposed as a possible predictive biomarker for FOLFIRI treatment outcome. In advanced colorectal cancer, two large retrospective studies investigating the relationship between Top1 protein levels and irinotecan treatment outcome produce conflicting results [8,9]. While these efforts have been directed at studying Top1 protein levels, research into chromosomal alterations involving the topoisomerase I gene (symbol: Top1) are limited. Located at 20q12-q13.1, part of the frequently gained 20q region implicated in adenoma to carcinoma progression [10–13], Top1 is found at elevated copy numbers in a large fraction of stage III CRC samples when detected by Fluorescent In Situ Hybridization (FISH) [14,15].

In our study of Top1, we have previously shown that in a stage III CRC chemonaive patient cohort (n = 154), increased Top1 gene copy number was significantly associated with longer survival (OS) [15]. Interestingly, an estimated 71% of patients harbored a Top1 gene copy increase, whereas only 10% of patients harbored a Top1 amplification [Top1/CEN-20 (centromere 20) Ratio ≥2.0] [15], indicating that gene amplification is not the most common mechanism for generating additional copies of Top1. A strong correlation between Top1 and CEN-20 was found, revealing an association between Top1 and CEN-20 copy number increases. This would suggest that gene gain mechanisms involving both the Top1 locus and the chromosome 20 centromeric region also occur, possibly by gain of the whole 20q arm by e.g. isochromosome formation or whole chromosome 20 gain (aneusomy). This type of gene copy number increase occurs by mechanisms related to chromosome missegregation and not gene amplification. Measuring gene copy number alterations by FISH traditionally relies on the use of a same chromosome reference probe, e.g. using CEN-20 for measuring genes on chromosome 20, we therefore set out to develop a novel FISH assay to distinguish tumor specimens with Top1 copy number increases due to amplifications from those with increases due to 20q gain or aneusomy by applying a reference probe directed at an unrelated chromosome.

The purpose of the current study is to determine the frequency of Top1 alterations, map any prognostic effects of these gene aberrations, identify cut-offs that reflect the underlying genetic mechanisms of Top1 copy number alterations and update FISH scoring guidelines to reduce observer workload. To achieve these goals, the mechanism of Top1 gene copy gain was investigated in a panel of CRC cell lines with the aim of identifying a reference probe that truly reflects ploidy levels, so that Top1 copy number increases should be detected in relation to the total number of chromosomes (ploidy level) and this is best done through the use of a gene to centromere ratio. A novel probe combination, consisting of Top1 and a centromere 2-specific (CEN-2) probe, was then applied to the previously tested stage III CRC patient samples to discriminate between patients harboring Top1 copy number increases caused by mechanisms involving chromosome missegregation and those caused by gene amplification. The relationship between the different mechanisms of Top1 copy number increase and patient prognosis was investigated. Additionally, based on all FISH data, we could update the Top1 FISH scoring guidelines.

Materials and Methods

2.1 Patients and Clinical Material

One hundred fifty-four CRC patients with histologically verified stage III adenocarcinomas and obtainable FFPE tumor specimens were selected as previously described (see Fig. 1) [15]. All patients had surgical resections of their CRC and received no adjuvant radio- and/or chemotherapy, as this was not part of standard CRC treatment in Denmark at the time (April 1991–August 1993). Patients were randomized to receive either Ranitidine or placebo for up to five years with no effect of ranitidine on survival reported [16]. Participants provided written informed consent and the study was conducted in accordance with the Helsinki II Declaration with approval from the Danish National Board of Health (2760-419-1989), Data Protection Agency (1991-1110-751) and Central National Ethics Committee (KF 01-2045/91). The approval included collection of tissue specimens for subsequent analysis of biological markers (KF 01-078/93).

2.2 Preparation of Metaphases & Non-truncated Interphase Nuclei

CRC cell lines Colo-205, HCC-2998, HCT-15, HCT-116, HT-29, KM12, and SW620 were obtained from the NCI/Development Therapeutics Program, while DLD-1, LoVo, and LS-174T were obtained from the American Tissue Culture Collection. The...
cells were maintained at 37°C, 5% CO₂ in RPMI 1640 GlutaMAX™ growth medium (Invitrogen, Carlsbad, USA) supplemented with 10% fetal calf serum (Invitrogen). Once cultures reached a confluence of ~70%, colcemid (Invitrogen) was added and cultures were incubated for 2 h at 37°C. Subsequently, cells were harvested and a hypotonic treatment was performed (0.075 M KCl) for 10 min. Fixation was performed (fixative: 3:1 vol/vol absolute methanol and glacial acetic acid) and this suspension was dripped onto glass slides.

2.3 TOP1/CEN-2 Probe Mixture

The TOP1 gene probe has previously been described elsewhere (K5499) and the Histology FISH Accessory Kit (K5799) (Dako). The CEN-2 probe (Dako, Glostrup, Denmark), consisting of several FITC-labeled peptide nucleic acid monomers directed at repetitive α-satellite sequences, was combined with the Texas Red-labeled DNA gene probe in the IQFISH buffer (Dako). The TOP1/CEN-20 probe combination has previously been described [14].

2.4 FISH Procedure

FISH reagents were from the Cytology FISH Accessory Kit (K5499) and the Histology FISH Accessory Kit (K5799) (Dako). Metaphase specimens were fixed in 3.7% formaldehyde, washed, dehydrated (in a 70%, 85%, 96% ethanol series) and air-dried. FISH probe was loaded onto slide, denatured at 82°C (TOP1/CEN-2: 5 min, TOP1/CEN-20: 10 min) and hybridized (TOP1/CEN-2: 1 h, TOP1/CEN-20: overnight). Excess probe was removed by washing in stringency buffer (TOP1/CEN-2: 63°C, TOP1/CEN-20: 65°C). Slides were washed, dehydrated, air dried and mounted. TOP1/CEN-2 FISH hybridization to FFPE specimens (thickness: 3 μm) was performed according to the manufacturer’s recommendations (Dako). Briefly, slides were heat pretreated (in microwave oven) and pepsin digested at 37°C. Slides were subsequently denatured at 66°C for 10 min and hybridized at 45°C for 1–2 h and thereafter treated as described above.

2.4.1 Scoring. FISH signals were initially scored as previously described [14]. Briefly, signals were counted in 60 non-overlapping nuclei if signals were, as a minimum, visible at 200× magnification in the appropriate filter. Scoring was performed at 1000× magnification in the Texas Red/FITC double filter. Signal counts were typed directly into an electronic scoring sheet (kindly provided by A. Schonau, Dako, Glostrup, Denmark). Initially, 60 nuclei were scored for each specimen following TOP2A FISH pharmDx™ guidelines (Code K5333, package insert, 1st edition, 2008.01.18) where nuclei harboring both signals, as well as nuclei harboring only reference signals were scored, which facilitates the detection of gene deletions. To improve assay sensitivity, only nuclei harboring both gene- and reference signals were included for further analysis.

To determine the mechanism of TOP1 copy number increase in cell lines, signal locations and numbers were noted for 50 metaphases for each cell line. The total number of chromosomes for each cell line was determined by taking digital images of three metaphases for each cell line and counting the total number of chromosomes manually.

To determine the haploid, diploid, triploid and tetraploid ranges for CEN-2, 60 nuclei were counted in the unaffected epithelium adjacent to tumor tissue. The diploid range was defined as follow: 2n = (n/2) [min] ≤ 2n < 2n + (n/2) [max], where 2n equals the mean CEN-2 counts per nucleus in the (diploid) unaffected epithelium. The triploid and tetraploid ranges were found by using 3n or 4n instead of 2n, respectively. The haploid and high ploidy level ranges were defined as below the diploid range and above the tetraploid ranges, respectively. Definition of ploidy ranges has previously been described [18].

2.5 Statistical Methods

All descriptive and survival analyses were performed by use of SAS 9.2 (SAS Institute, Cary, USA). R version 2.15.1 was used in scoring method optimization.

2.5.1 Scoring methods. Gene to centromere ratios were calculated by including either the first 10 or 20 nuclei, determining TOP1 status and comparing this to the status after inclusion of all relevant nuclei. Concordance was calculated by use of Kendall’s tau [tau = (agree-disagree)/(agree + disagree)]. Borderline intervals near the cut-off values, where additional nuclei must be included (for 10 nuclei, an additional 10 nuclei had to be scored; for 20 nuclei, an additional 20 nuclei had to be scored) were defined as greater than or equal to 1.35 (min) and less than 1.65 (max) when applied cut-off was 1.5. Using HER2/CEN-17 guidelines, the borderline interval covering the cut-off of 2.0 was defined as greater than or equal to 1.8 (min) and less than 2.2 (max) [19]. Concordance and mean number of nuclei scored were calculated with and without borderline intervals.

2.5.2 Survival analysis. Three endpoints were considered: overall survival (OS, time to death by any cause), time to recurrence (TTR, time to any event related to colorectal cancer) and time to local recurrence in rectal cancer (LR) (described in detail in [15]). Kaplan-Meier estimates of survival probabilities are presented for the binary variables and some combinations. Multivariable analysis was done adjusting for gender, age (per 10 year difference in age) and tumor localization (RC versus CC). Cox regression analysis was used for the analyses. The models were validated by assessing the proportionality assumption and linearity for continuous covariates employing Schoenfeld and Martingale residuals. TOP1 and CEN-2 copy numbers, when analyzed as a continuous variable were log transformed (base 2) and therefore reflected a two-fold difference for these variates. Results are presented by hazard ratios (HR) with 95% confidence intervals (CI) and p-values. All calculated p-values were two-sided and considered significant at 0.05.

3 Results

3.1 Mechanisms of TOP1 Gene Copy Increase in Cell Line Panel

To determine the underlying mechanism(s) of TOP1 gene copy number increase, metaphase spreads were prepared from a panel of ten CRC cell lines. Metaphase preparation was successful for all but one of the cell lines (LS-174T). Following subsequent hybridization with the TOP1/CEN-20 probe, metaphase spreads were analyzed with regards to total number of chromosomes, the number of gene- and centromere-signals, as well as signal location, the results of which can be viewed in Table 1 and Figure 2. TOP1 gene copy number increases were observed in four of the nine cell lines. In both Colo-205 and SW620, gene gain appeared to be linked to chromosome 20 aneusomy (Fig. 2A and 2D, respective). In HT-29 (Fig. 2B), TOP1 gene gain occurred in a fashion suggestive of 20q isochromosome formation. In KM12, TOP1 gain occurred independently of CEN-20 (Fig. 2C). No TOP1 amplifications were observed. As shown in Table 1, only TOP1 gene gains which do not involve CEN-20 (in a 1:1 fashion) are reflected in the TOP1/CEN-20 ratio.

3.2 Identification of a New Reference Probe

To identify a relevant marker for cellular ploidy, i.e. the total number of chromosomes, the NCI and NCI-Bi's SKV/M-FISH
and CGH Database (http://www.ncbi.nlm.nih.gov/sky/skyweb.cgi) was screened. Chromosome 2 appeared to be the least affected by independent numeric aberrations, such as whole chromosome gain, and was therefore selected for further analysis in the cell line metaphase panel. As shown in Table 1, triploid cell lines (Colo-205 and HT-29) were found to produce three CEN-2 signals, while the diploid cell lines produced only two. All TOP1 gene copy increases were reflected in the TOP1/CEN-2 ratio using a cut-off value of 1.5, representing a 3:2 situation between gene and centromere and reflecting an additional TOP1 copy in a diploid cell.

3.3 Stage III CRC Patient Material

TOP1/CEN-2 FISH hybridization and evaluation was successful for 151 of 154 patient FFPE tumor samples (98%) (see Fig. 1). The distribution of TOP1 and CEN-2 signals was homogenous in tumor specimens. To improve upon sensitivity to detect specimens harboring additional copies of TOP1, only nuclei harboring both TOP1 and CEN-2 signals were included in subsequent analysis, which resulted in a median of 58 nuclei scored for each tumor specimen (range: 47–60). In 50 randomly selected samples, CEN-2 signals were counted in the unaffected colon mucosa to determine mean signals counts (mean: 1.37, median: 1.38, range: 1.20–1.62). These counts were used to define the diploid range (see section 2.4.1).

In the tumor material, CEN-2 ranged from 1.19 to 2.52 with a median of 1.70. By comparing mean CEN-2 signals counts from the tumor nuclei to the non-tumor nuclei, the majority (97.4%) of tumor samples could be classified as harboring two (disomic) or three (trisomic) copies of chromosome 2 (Table 2). In the tumor samples, TOP1 signals ranged from 1.33 to 6.72 per nucleus with a median of 3.17 signals while the TOP1/CEN-2 ratio ranged from 1.01 to 3.39 with a median of 1.92. No deletions (TOP1/CEN-2<0.8) were observed.

3.3.1 Determining TOP1 status. To identify samples harboring a TOP1 gene copy number increase, a TOP1/CEN-2 ratio cut-off of 1.5 was applied. As shown in Fig. 3, samples producing ratios equal or above this cut-off received the TOP1 status ‘Gain’, whereas those below were termed ‘TOP1 Normal’. Initially, 103 patients (68.2%) were classified as ‘Gain’ using this cut-off.

Once tumor specimens harboring an additional copy of TOP1 were identified, data on TOP1/CEN-20 was used to elucidate the mechanism of TOP1 gene copy increase. By applying a TOP1/CEN-20 ratio cut-off of 2.0 to distinguish between samples where TOP1 gene gain occurs independently of CEN-20 (TOP1/CEN-20≥2.0) from those where gene gain occurs due to aneusomies or 20q isochromosome formation (TOP1/CEN-20<2.0), specimens with a TOP1 gain could be further dichotomized into amplified and non-amplified subgroups. Therefore, samples producing a TOP1/CEN-2 ratio of equal or above 1.5 and a TOP1/CEN-20 ratio above or equal to 2.0 were termed ‘TOP1 Amplification’, whereas for those below 2.0 were given a ‘TOP1 Non-amplified

Table 1. CRC cell line stemline populations.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Number of Chromosomes</th>
<th>Ploidy level</th>
<th>TOP1 Count</th>
<th>CEN-20 Count</th>
<th>CEN-2 Counts</th>
<th>TOP1/CEN-2 Ratio</th>
<th>TOP1/CEN-20 Ratio</th>
<th>TOP1/CEN-20 Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colo-205</td>
<td>71–72</td>
<td>Near Triploid</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>1.00</td>
<td>1.67</td>
<td></td>
</tr>
<tr>
<td>DLD-1</td>
<td>45–47</td>
<td>Near Diploid</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>HCC-2998</td>
<td>46–49</td>
<td>Near Diploid</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>HCT-116</td>
<td>45–48</td>
<td>Near Diploid</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>HCT-15</td>
<td>44–46</td>
<td>Near Diploid</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>HT-29</td>
<td>68–72</td>
<td>Near Triploid</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>1.25</td>
<td>1.67</td>
<td></td>
</tr>
<tr>
<td>KM12</td>
<td>43–44</td>
<td>Near Diploid</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1.50</td>
<td>1.50</td>
<td></td>
</tr>
<tr>
<td>LoVo</td>
<td>46–47</td>
<td>Near Diploid</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>SW620</td>
<td>43–49</td>
<td>Near Diploid</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>1.00</td>
<td>1.50</td>
<td></td>
</tr>
</tbody>
</table>

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Gain’ status (see Fig. 3). As shown in Table 3, the majority (58.4%) of tumor specimens received a TOP1 Non-amplified Gain status. All samples classified as TOP1 Amplification were also found to produce a TOP1/CEN-2 ratio of above 2.0 (see Table 3).

To verify the categorization of samples into subgroups, mean CEN-2 and CEN-20 signals in tumor nuclei were compared to their respective means in unaffected colon mucosa, the results of which are listed in Table 2. Samples with mean CEN-2 and CEN-20 in the diploid range belonged in 21/34 (61.8%) cases to the TOP1 Normal category. Near-triploid and CEN-20 aneusomic cases were most often found in the samples classified as TOP1 Non-amplified Gain. CEN-2 aneusomy was observed in 19 (12.6%) cases.

3.3.2 Association with patient outcome. The relationship between biomarker status and patient outcome was explored in both univariate and multivariate models. In this patient cohort, there were 112 deaths of all causes and 88 recurrences including cancer-specific deaths within five years [15]. TOP1 copy number,

Table 2. Definition of ploidy ranges from unaffected colon mucosa specimens and estimation of ploidy in tumor samples.

<table>
<thead>
<tr>
<th>Ploidy Status</th>
<th>CEN-2 copy number/Whole nuclei</th>
<th>CEN-2 copy number/Truncated nuclei</th>
<th>Frequency of samples with CEN-20 aneusomy (CEN-20&gt;CEN-2)b</th>
<th>Frequency of samples with CEN-2 aneusomy (CEN-2&gt;CEN-20)b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Range</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haploid/Monosomy</td>
<td>1</td>
<td>0.69</td>
<td>&lt;1.03</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Diploid/Disomy</td>
<td>2</td>
<td>1.37</td>
<td>[1.03–1.72)</td>
<td>79 (52.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>45 (29.6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Triploid/Trisomy</td>
<td>3</td>
<td>2.06</td>
<td>[1.72–2.41)</td>
<td>68 (45.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>26 (17.2)</td>
<td>17 (11.3)</td>
</tr>
<tr>
<td>Tetraploid/Tetrasomy</td>
<td>4</td>
<td>2.75</td>
<td>[2.41–3.09)</td>
<td>4 (2.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 (0.7)</td>
<td>2 (1.3)</td>
</tr>
<tr>
<td>High ploidy level</td>
<td>&gt;5</td>
<td>-</td>
<td>3.09</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

a% denotes the percentage of specimens in category relative to the total number of specimens (151).
bCEN-20 aneusomy was defined as specimens with CEN-20 signals in a higher ploidy range than CEN-2. CEN-2 aneusomy was similarly defined, although vice versa.

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Figure 3. Types of TOP1 gene copy number increases by TOP1 status. Red line denotes TOP1 gene signal and green dot denotes centromeric signal. Examples are based on CRC cell line metaphase results – trisomy (SW620), pentasomy (Colo-205), 20q isochromosome formation (HT-29) and 20q gain (KM12).

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by itself, was not significantly associated with OS, TTR or LR (see Table 4). Higher CEN-2 copy numbers were associated with better prognosis with OS as clinical endpoint in the multivariate analysis (HR: 0.38, 95% CI: 0.15–0.98, p = 0.04), while only a tendency was observed in the univariate analysis (HR: 0.49, see Table 4).

Initially, patients harboring TOP1 increases (TOP1 Gain, see Fig. 3) were compared to those without (TOP1 Normal). Gain of TOP1 was not significantly associated with OS or TTR in both the univariate and multivariate analysis (see Table 4). Patients with TOP1 amplifications (TOP1/CEN-2=2.0) were initially compared to non-amplified cases (TOP1 Normal and TOP1 Non-amplified Gain subgroups combined). Amplification of TOP1 was not significantly associated with OS or TTR, although approached significance for TTR in the multivariate analysis (HR: 0.50, 95% CI: 0.23–1.09, p = 0.08). Analysis of TOP1 amplifications in relation to LR failed due to a very limited number of events. Additional cut-offs for both probe combinations were investigated and the results are listed in Table 4.

Following the primary analysis of data, specimens were stratified in subgroups depending on the presence and type of TOP1 increase (see Fig. 3, listed in Table 3). As shown in Table 5, no significant difference was observed between the TOP1 Normal, TOP1 Non-amplified Gain and TOP1 Amplification subgroups with OS as endpoint in both the univariate and multivariate analysis. With TTR as clinical endpoint, TOP1 Non-amplified gains showed a tendency towards shorter TTR (HR: 1.57, 95% CI: 0.97–2.55, p = 0.07) in the univariate analysis, and a similar, but weaker, tendency observed in the multivariate analysis (HR: 1.49). TOP1 amplifications did not exhibit any significant relationship to TTR when compared to the TOP1 Normal baseline subgroup. A Kaplan-Meier plot for these relationships can be viewed in Fig. 4B.

### 3.4 TOP1 Scoring Guidelines

The possibility of scoring fewer nuclei in the determination of TOP1 status presents an opportunity to diminish the observer workload. To determine whether this was possible, status of TOP1/CEN-2 and TOP1/CEN-20 was determined using 10 or 20 nuclei and compared with the ratio status after the inclusion of all relevant nuclei. As shown in Table 6, scoring a reduced number of nuclei, such as only 10 or 20 nuclei to determine TOP1/CEN-2 status (cut-off 1.5) classified samples with moderate concordance (0.76 and 0.91, respectively), which increased with the introduction of borderline intervals, where additional nuclei must be scored (see section 2.5.1). For the detection of TOP1 amplifications (cut-off: 2.0), concordance was relatively high (10 nuclei: 0.96, 20 nuclei: 0.99) and was not improved with the use of borderline intervals. Additionally, alternative cut-offs were investigated, the results of which are listed in Table 6.

### Discussion

#### 4.1 Mechanisms of TOP1 Copy Number Increase

In this study, four different mechanisms of TOP1 copy number increase were identified. In the cell line panel mechanisms involving TOP1 and CEN-20 (Colo-205, HT29 and SW620) as well as a mechanism where TOP1 was gained independently of CEN-20 (KM12), were observed. In Colo-205 and SW620,
Chromosome 20 aneusomy was observed, in agreement with the NCI and NCBI’s SKY/M-FISH and CGH Database (http://www.ncbi.nlm.nih.gov/sky/skyweb.cgi). This type of increase transpires due to the missegregation of chromosomes during mitosis, resulting in an abnormal number of chromosomes; a karyotypic state termed ‘aneuploidy’.

In HT29, TOP1 gain occurred in a manner suggestive of the formation of an isochromosome, in line with other results [20]. This mechanism of TOP1 gene copy number increase occurs owing to a misdivision of the centromere (transverse breakage, rather than longitudinal) during chromosome segregation, resulting in a chromosome with two identical arms. In KM12, an additional TOP1 signal was observed on a chromosome which did not harbor a CEN-20 signal. In the NCI and NCBI’s SKY/M-FISH and CGH Database, this cell line appears to harbor a fusion chromosome of 22q and an additional copy of 20q, where CEN-20 (or at least the alpha-satellite sequences targeted by the CEN-20 probe) was not gained along with the rest of 20q. This type of gain may be the product of chromosome 20 aneusomy followed by a Robertsonian translocation event.

While gene copy number increase can occur due to events involving larger chromosomal regions, such as the gain of whole chromosomes or chromosomal arms, it can also occur due to gene amplification. Gene amplification has been proposed to occur through several mechanisms, including errors in DNA replication and repeated breakage-fusion-bridge cycles due to double-strand DNA break or telomere dysfunction [21–23]. A chromosomal region preferentially gained through amplification is termed an ‘amplicon’, an approximately 0.5–10 Mb DNA fragment in length, and usually encompasses gene(s) involved in promoting tumor growth [24]. It should be noted that whole chromosome or chromosome arm alterations generally occur more frequently, but in lower magnitude, than to smaller chromosomal alterations [25].

In the present study, TOP1 gene amplification was defined as TOP1/CEN-20 ratio equal or above 2.0. This definition means that TOP1 exists at levels double that of its host chromosome. Therefore, this type of gene copy increase is probably due to the copy number increase of an amplicon and not arm-length chromosomal regions, since its mechanism of copy number increase is independent of CEN-20. No TOP1 amplifications were observed in the nine cell lines investigated, but was detected in 10% of tumor samples. This could suggest that either this mechanism is patient-specific or that this mechanism was not present in our limited number of cell lines investigated.

While each of the aforementioned mechanisms of TOP1 gene copy number increase occur as single events in cell lines, our results indicate that they can occur simultaneously in tumor specimens. In 4/15 (26.7%) cases of amplification (data not shown), CEN-20 aneusomy was detected, indicative of an amplification mechanism, as well as one involving aneusomy or isochromosome formation. In samples classified as TOP1 Non-amplified Gain, it is possible that both 20q isochromosomes and additional copies of chromosome 20 are present. However, it is only possible to classify specimens according to predominant mechanism.

Table 5. TOP1 Status and association to prognosis.

<table>
<thead>
<tr>
<th>Clinical Endpoint</th>
<th>TOP1 Status</th>
<th>Univariate HR</th>
<th>95% CI</th>
<th>p-value</th>
<th>Multivariate* HR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS</td>
<td>Normal</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Non-amplified Gain</td>
<td>1.01</td>
<td>0.68</td>
<td>1.52</td>
<td>0.95</td>
<td>1.08</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>Amplification</td>
<td>0.61</td>
<td>0.30</td>
<td>1.22</td>
<td>0.16</td>
<td>0.62</td>
<td>0.31</td>
</tr>
<tr>
<td>TTR</td>
<td>Normal</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Non-amplified Gain</td>
<td>1.57</td>
<td>0.97</td>
<td>2.55</td>
<td>0.07</td>
<td>1.49</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>Amplification</td>
<td>0.73</td>
<td>0.31</td>
<td>1.70</td>
<td>0.47</td>
<td>0.66</td>
<td>0.28</td>
</tr>
</tbody>
</table>

*Adjusted for age, gender and tumor localization.

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Figure 4. Kaplan-Meier plots illustrating patient outcome according to TOP1 status. A (left): OS, B (right): TTR.

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4.2 Role of 20q

Gain of chromosome 20 or 20q has been widely reported as a recurrent chromosomal abnormality in colorectal cancer [11,28–33], supporting the high frequency of TOP1 non-amplified gains observed in this study. In vitro models suggest that 20q gain plays a causative role in tumorigenesis, as well as in increasing cellular proliferation rates [34]. In colorectal cancer, 20q is believed to play a role in colorectal adenoma to carcinoma progression [10–13] and is often observed in tumors exhibiting the microsatellite stable and/or chromosomal instability phenotypes [32,35,36]. Numerous genes located on 20q have been reported to have altered expression as a consequence 20q gain, including several well-known cancer-related genes, such as AURKA (20q13.1) [11], BCL2L1 (20q11.21) [25] and AIB1 (20q12) [27]. TOP1 mRNA expression has not been reported to be affected by copy number increases, whereas PLCG1, a neighboring gene (and partly covered by the TOP1 FISH probe), copy number increases have been found to correlate significantly with expression [27]. It should be noted that PLCG1 appears to be involved in tumorigenesis [37].

4.3 Centromere 2 Versus Centromere 20

Following the analysis of TOP1/CEN-20 in this patient cohort [15], it became evident that TOP1 underwent copy number increases in conjunction with CEN-20 in a large fraction of specimens. Therefore, CEN-20 was deemed to be an inappropriate marker for cellular ploidy levels. In our analysis of the metaphase cell line panel, CEN-2 was found to correctly reflect the total number of chromosomes and thus the ploidy levels. Chromosome 2 has not been reported to undergo above background numeric aberrations in CRC [11,28–33,35]. In a landmark study, consisting of 3138 cancer specimens from 26 different cancer types, 2p and 2q were shown to undergo significantly fewer arm-level alterations when compared to other chromosomes [25]. Using the Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer (http://cgap.nci.nih.gov/Chromosomes/Mitelman) to investigate the frequency of numerical aberrations involving chromosome 2 in tumors of the large intestine, 55 cases were identified from a total of 524 (consisting of adenomas, adenocarcinomas and carcinomas of the large intestine), a frequency of 10.3%. Of these 55 cases, 29 would not result in incorrect TOP1 status; 20 would result in false negatives; 3 would result in false positives; and a single case not providing sufficient karyotypic information to determine TOP1 status. Overall, CEN-2 would allow correct TOP1 status categorization in 95.2% of samples. These findings support the use of CEN-2 as a reference probe to measure cellular ploidy levels in tumors of the large intestine.

A comparison of CEN-2 and CEN-20 signals in tumor nuclei revealed a high frequency of CEN-20 aneusomy (47.6%), supporting the presence of additional copies of chromosome 20 or 20q. CEN-2 aneusomy was detected in 19 specimens (12.6%). It should be noted that these findings are merely estimations, as ploidy ranges were defined by the mean signal counts in unaffected colon epithelium by two different observers. In normal tissue, mean CEN-20 was found to be 1.57 [15], whereas CEN-2 was 1.37, a discrepancy that may be attributed to interobserver variability and illustrates the potential pitfall of using mean signal counts in the analysis of FISH data.

4.4 Association to Patient Outcome

We have previously reported that higher TOP1 gene copy numbers, when analyzed as a continuous variable, were associated with improved patient outcome in this particular patient cohort [15]. With OS as endpoint, TOP1 increases were previously reported to be significantly associated with longer survival in both the univariate (HR: 0.71, 95% CI: 0.50–0.99, p = 0.04) and multivariate analysis (HR: 0.62, 95% CI: 0.42–0.90, p = 0.01) [15]; however, these findings could not be reproduced in the current study. This suggests that the previously reported findings regarding OS and TTR may have been observer dependent. The TOP1 Gain status, by itself, was not associated with altered OS, TTR or LR, which may be due to the inclusion of TOP1 amplified specimens. Once amplified cases were segregated from cases of TOP1 gain, a trend toward shorter TTR was observed for TOP1 non-amplified gains (see Table 5). Amplification of the TOP1 gene, representing a situation where CEN-20 is not involved in gene gain, exhibited a weak trend towards longer TTR when compared to the rest of the population, a finding which may be attributed to the low number of event in this particular subgroup. When compared only to the TOP1 Normal subgroup, this effect diminished. While TOP1 Non-amplified gains showed a tendency towards poor prognosis (with TTR as endpoint), TOP1 amplification displayed a weak trend towards better prognosis (with OS as endpoint), producing non-significant hazard ratios below 1.0 (see Fig. 4B, table 5). It is unknown what effect the different TOP1 gain mechanisms have on protein expression and why they may harbor opposite prognostic impacts. It should be noted that only 15 specimens (10%) were amplified, a low amount for a study of modest size. This observation requires validation in a larger patient cohort.

Table 6. Characteristics of the updated scoring guidelines.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Ratio cut-off</th>
<th>Borderline interval</th>
<th>Concordance Mean number of scored nuclei</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>10 nuclei 20 nuclei</td>
</tr>
<tr>
<td>TOP1/CEN-2</td>
<td>1.5</td>
<td>none</td>
<td>0.76 0.91</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>1.35–1.65</td>
<td>0.92 1.00</td>
</tr>
<tr>
<td>TOP1/CEN-20</td>
<td>2.0</td>
<td>none</td>
<td>0.70 0.80</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>1.80–2.20</td>
<td>0.88 0.95</td>
</tr>
<tr>
<td>TOP1/CEN-20</td>
<td>1.5</td>
<td>none</td>
<td>0.82 0.87</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>1.35–1.65</td>
<td>0.96 0.99</td>
</tr>
<tr>
<td>TOP1/CEN-20</td>
<td>2.0</td>
<td>none</td>
<td>0.96 0.99</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>1.80–2.20</td>
<td>0.96 0.99</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0060613.t006
In the present study, higher CEN-2 counts were significantly associated with longer survival in the multivariate analysis (HR: 0.36, 95% CI: 0.15–0.94, p = 0.04). This suggests longer survival time for patients with tumors with a higher total number of chromosomes, i.e. near-triploidy and -tetraploidy, which stands in contrast to previously published results, which describe near-triploid karyotypes being associated with shorter survival when compared with near-diploid ones in CRC [38]. It should be noted that CEN-20 aneusomy was detected to a lesser extent at higher ploidy levels (see Table 2), opening the possibility that the association between CEN-2 and survival may be attributed to this. Alternatively, aneusomy of chromosome 2 (estimated to occur in 12.6% of specimens, detected in near-triploid and near-tetraploid tumors) may contribute to an undocumented prognostic value of chromosome 2 numeric aberrations. In any case, this result requires further validation in another patient cohort.

It is unknown which types of TOP1 gene copy increase mechanisms, if any, yield tumors responsive or resistant to Top1 inhibitor treatment, such as irinotecan. The present study has several limitations, including a patient population treated with outdated surgical techniques and no adjuvant therapy and should merely serve as an explorative study of the types of TOP1 gene copy increases present in CRC and how these can be detected by FISH. Predictive biomarkers often harbor a prognostic component, a feature which may potentially overshadow the beneficial effects of a given treatment unless the prognostic element has been investigated [39]. While a limited number of significant relationships were identified in this study, we believe that the findings are relevant to report as they may aid future study design to determine whether TOP1 gene aberrations hold any predictive value in relation to TOP1 inhibitor-based chemotherapy. We observed that TOP1 non-amplified gains showed a non-significant tendency toward poorer prognosis, which may be attributed to increased expression of other cancer-related genes on 20q (see section 4.2).

The prognostic value of amplification, which appeared to be positive, could not be clearly determined due to the low number of events. CEN-2 aneusomy occurred at levels lower than CEN-20, suggesting that it is more suitable as a reference probe to detect ploidy levels, as supported by the findings from the CRC metaphase panel. As no deletions were observed (TOP1/CEN-2<0.8), we can conclude that high level CEN-2 aneusomy does not occur independently of CEN-20 gain. The significant relationship between longer survival and higher CEN-2 counts should be interpreted with caution, as only two samples were in the tetraploid range and this relationship was not observed with other clinical endpoints. We therefore propose that CEN-2 be used in combination with other reference probes to elucidate the mechanism of gene gain, which may have relevance in other studies of copy number alterations by FISH. Future plans for TOP1 FISH include testing both probe combinations in a retrospective material with a suitable number of irinotecan treated patients and relevant controls to determine whether any of the TOP1 status subgroups respond to irinotecan. Furthermore, a probe set including both centromere probes in different colors will be considered to reduce observer workload.

4.5 Updated Guidelines

In order to reduce observer workload, the possibility of determining TOP1 status by scoring fewer nuclei was investigated. As shown in Table 6, using a borderline interval achieved improved concordance when compared to scoring without using a borderline interval. For the validation of a HER2 assay, Wolff et al. [19] suggest a minimum of 95% concordance with an alternative validated method or same validated method, a requirement which was adopted in the current study. For TOP1/CEN-2 (cut-off 1.5), this requirement was surpassed by the inclusion of 20 nuclei with additional nuclei scored if the ratio was within the relevant borderline interval after 20 nuclei. This resulted in an average of 24.8 nuclei scored for each sample with these updated guidelines. For TOP1/CEN-20 (cut-off 2.0), scoring 10 nuclei was sufficient to achieve a concordance of 0.96. For practical purposes, we have chosen to score 20 nuclei with relevant borderline intervals to determine TOP1 status, thereby reducing the amount of scored nuclei by 58.7 and 64.7 percent for TOP1/CEN-2 (cut-off 1.5) and TOP1/CEN-20 (cut-off 2.0), respectively (see mean nuclei scored, Table 6). We therefore propose that future use of TOP1 FISH in CRC is based upon scoring 20 nuclei containing both gene- and reference signals and using the aforementioned borderline intervals, where an additional 20 nuclei must be scored. TOP1 status could not be determined by use of the TOP1 gene probe by itself in a reproducible manner (data not shown).

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Author Contributions

Conceived and designed the experiments: DHS KVN. Performed the experiments: DHS NEJ. Analyzed the data: DHS IJC BM. Contributed reagents/materials/analysis tools: SM HJN NB NFJ MUR SBN. Wrote the paper: DHS KVN.

References


