Evaluation of the correlation between *KRAS* mutated allele frequency and pathologist tumorous nuclei percentage assessment in colorectal cancer suggests a role for zygosity status

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ABSTRACT

Evaluation of molecular tumour heterogeneity relies on the tumorous nuclei percentage (TNP) assessment by a pathologist, which has been criticised for being inaccurate and suffering from interobserver variability. Based on the 'Big Bang theory' which states that *KRAS* mutation in colorectal cancer is mostly homogeneous, we investigated this issue by performing a critical analysis of the correlation of the *KRAS* mutant allele fraction with the TNP in 99 colorectal tumour samples with a positive *KRAS* mutation status as determined by next-generation sequencing. Our results yield indirect evidence that the KRAS zygosity status influences the correlation between these parameters and we show that a well-trained pathologist is indeed capable of accurately assessing TNP. Our findings indicate that tumour zygosity, a feature which has largely been neglected until now, should be taken into account in future studies on (colorectal) molecular tumour heterogeneity.

The Big Bang theory in colorectal cancer states that *KRAS* mutations are an early event in carcinogenesis and therefore mutations in this driver gene are frequently present throughout the tumour in a homogeneous fashion, while mutations in other genes such as *BRAF* and *PIK3CA* occur later and therefore these tend to be present heterogeneously.[1 2] The studies that have led to this hypothesis are based on and take into account assessment of tumorous cell, or rather nuclei, percentage by a pathologist, since tumorous samples by definition do not contain tumorous nuclei and nuclei from non-tumorous cells such as myofibroblasts and lymphocytes. However, several studies have put forward that the assessment of tumorous nuclei percentage (TNP) by pathologists is not very accurate, showing considerable interobserver variability.[3 4]

To further investigate this issue, we performed a critical analysis of the correlation between *KRAS* mutated allele frequency (MAF) and TNP assessed by a pathologist (PB) in 99 colorectal cancer samples (primary tumours and metastatic sites in 89 and 10 patients, respectively) that had a positive *KRAS* mutation status as determined by next-generation sequencing (NGS) performed between January 2016 and June 2017. Scoring by the pathologist was done *by eye balling* using intervals of 10% and NGS was performed using the human tumour actionable mutation panel from Qiagen, which includes the *KRAS* gene, on an Illumina MiSeq.

Mutations affecting exon 2 codons 12 and 13 accounted for 66% and 16%, respectively, of all *KRAS* mutations found, in line with published positivity rates of different types of *KRAS* mutations in European patients with colorectal cancer .[5] As shown in Figure 1, there is a significant correlation between *KRAS* MAF and TNP, with a Pearson correlation coefficient of 0.3881 and a p value of 0.000072. By evaluating this graph and by looking at the raw data, with a MAF of more than 50% in several tumours suggesting that at least some tumours are not heterozygous, we speculated that differences in zygosity status might explain why this correlation, albeit present, was not very strong and why some tumours showed a TNP that was about the double of the MAF. For example, patient number 44 had a tumour with a MAF of 23% and a TNP of 50%, while patient number 75 had also a MAF of 23% but a TNP of 20%. Assuming that the former tumour is heterozygous for the *KRAS* mutation, the MAF has to be doubled to compensate for the heterozygosity which cannot be seen by the pathologist and the resulting new MAF is 46%, which is close to identical to the TNP with a difference of only 4%, while the difference before this adjustment was 27%. The latter tumour is assumed to be homozygous for the *KRAS* mutation, hence the MAF is not changed and the difference between the MAF and the TNP therefore remains 3%. We re-evaluated the MAF of all tumours and when the difference between the doubled MAF and the TNP was smaller than between the original MAF and the TNP, the doubled MAF was used as the new MAF and the tumour was considered as being heterozygous. By doing this, we assigned a heterozygous and homozygous status to 30% and 70% of tumours, respectively. The new p value was <0.00001 and the new correlation coefficient was 0.6912, being almost the double of the original correlation coefficient. Compared with figures 1 and 2 indeed clearly depicts a much stronger correlation between the *KRAS* MAF and the TNP.

Our findings suggest that heterogeneity of *KRAS* mutation in colorectal cancer might actually even be less frequent than currently thought, because heterogeneity is deducted when a strong discrepancy between the MAF and the TNP is observed.[2 6] However, difference in zygosity status

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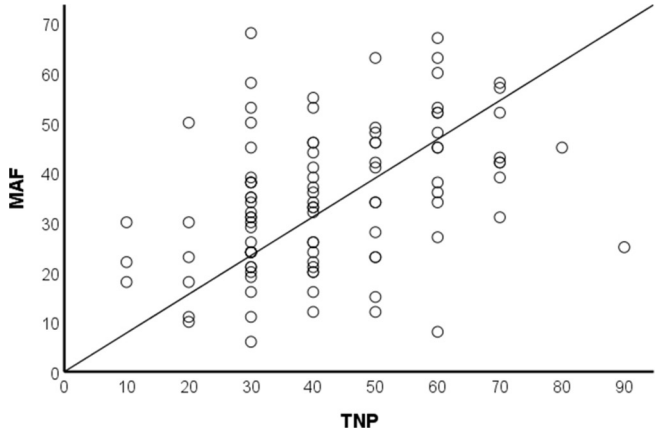

Figure 1 X–Y scatter plot showing the correlation between the tumorous nuclei percentage (TNP) and the *KRAS* mutant allele frequency (MAF).

between tumours might lead to a false impression of discrepancies. Therefore, our findings can be seen as a confirmation of the Big Bang theory. Moreover, our findings clearly show that a pathologist, even without the use of an zygosity status adjusted MAF, is indeed capable of performing a rather accurate assessment of TNP in a sample, in contrast with previous publications that were based on comparisons between pathologists, and not between a pathologist and the molecular data obtained from the samples.[3 4] Thus, it is indeed useful to include TNP assessments in external quality control schemes.

A *KRAS* mutation zygosity analysis of 137 cell lines, including several of colorectal origin, showed that 37% was homozygous[7]; in vivo data on the prevalence of homozygosity in colorectal cancer are currently not available. The assumption that colorectal tumours are per definition heterozygous and therefore the MAF for all tumours can be doubled when correlation with TNP is done *since each nucleus contains two alleles*[2 6]

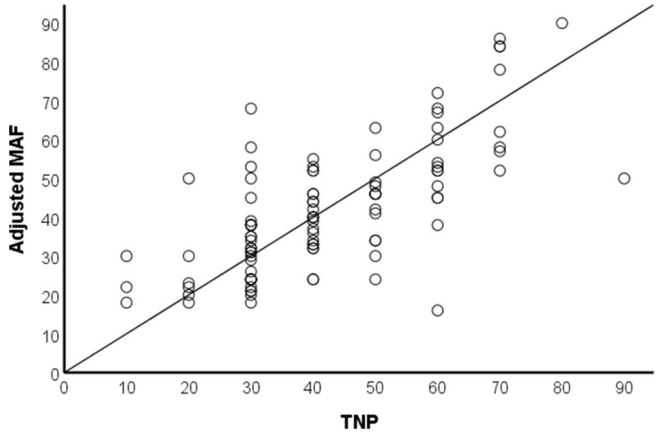

Figure 2 X–Y scatter plot showing the correlation between the tumorous nuclei percentage (TNP) and the adjusted *KRAS* mutant allele frequency (MAF) (see text for adjustment methods).

should clearly be re-evaluated *based on our data*. Furthermore, *KRAS* mutation copy number gains due to amplification or polysomy *or mutant allelic specific imbalance* could also play a role,[7] but if *these phenomena* would be present at a significant level in colorectal cancer, it would be impossible to find our reported correlation between *KRAS* MAF and TNP, either with or without the correction for presumed zygosity. Although we acknowledge that some assumptions are made and that our evidence is indirect, we feel that our findings are strong enough to propose that further research should address the issue of the zygosity status of *KRAS* and other mutations in colorectal cancer. Our findings could have consequences regarding the concept of heterogeneity in colorectal cancer and regarding the discussion on the accuracy of the pathologist's assessment of TNP in a sample.

Take home messages

- The data show that a well-trained pathologist is capable of accurately assessing tumoral nuclei percentage in the setting of colorectal cancer, supporting the use of such assesments in external quality assessment schemes.
- We find that the zygosity status of KRAS mutation probably influences the correlation between the KRAS mutant allele percentage and the tumoral nuclei assessment by the pathologist.
- Therefore, tumor zygosity, a feature which has largely been neglected until now, should be taken into account in future studies on (colorectal) molecular tumor heterogeneity.

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Competing interests None declared.

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REFERENCES

1 Sottoriva A, Kang H, Ma Z, *et al*. A Big Bang model of human colorectal tumor growth. *Nat Genet* 2015;47:209–16.
2 Normanno N, Rachiglio AM, Lambiase M, *et al*. Heterogeneity of KRAS, NRAS, BRAF and PIK3CA mutations in metastatic colorectal cancer and potential effects on therapy in the CAPRI GOIM trial. *Ann Oncol* 2015;26:1710–4.
3 Viray H, Li K, Long TA, *et al*. A prospective, multi-institutional diagnostic trial to determine pathologist accuracy in estimation of percentage of malignant cells. *Arch Pathol Lab Med* 2013;137:1545–9.
4 Smits AJ, Kummer JA, de Bruin PC, *et al*. The estimation of tumor cell percentage for molecular testing by pathologists is not accurate. *Mod Pathol* 2014;27:168–74.
5 Boleij A, Tack V, Taylor A, *et al*. RAS testing practices and RAS mutation prevalence among patients with metastatic colorectal cancer: results from a Europe-wide survey of pathology centres. *BMC Cancer* 2016;16:825.
6 Haley L, Tseng LH, Zheng G, *et al*. Performance characteristics of next-generation sequencing in clinical mutation detection of colorectal cancers. *Mod Pathol* 2015;28:1390–9.
7 Soh J, Okumura N, Lockwood WW, *et al*. Oncogene mutations, copy number gains and mutant allele specific imbalance (MASI) frequently occur together in tumor cells. *PLoS One* 2009;4:e7464.