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# Letter to the Editor

## KRAS transitions and villous growth in colorectal adenomas

To the Editor:

Colorectal cancer (CRC) is the second most common malignancy in the western world with a lifetime risk in the general population of about 5%. The genesis of CRC and its progression, mainly from pre-existing sporadic adenomas, are caused by somatic mutations of several genes including *APC*, *KRAS*, *SMAD4*, *TP53*, and *β-catenin*, by epigenetic events leading to gene activation and silencing, and by a large number of chromosomal aberrations leading to gene inactivation, amplification, and to more subtle gene dosage changes [7, 8,10,16,20].

The *KRAS* gene, in particular, one of the oldest oncogenes, is apparently having a second youth. The role of *KRAS* in the genesis and progression of CRC has been recently focused in a series of reviews linking the constitutive activation of this oncogene with a complex network of interactions leading to cell survival, apoptosis, angiogenesis, invasion and metastasis [17, 25,34]. An additional link of *RAS* mutations and chromosomal aberrations, a bona fide consequence of chromosomal instability (CIN), was suggested by a large series of *in vitro* and *in vivo* studies and during the human colorectal adenoma to carcinoma transition [5].

Though the mechanisms linking genetic and epigenetic events with CIN still remains unclear, it is commonly thought that CIN and aneuploidy represent early key genomic events in the genesis and progression of CRC and of other tumor types [4,8,9,16,21,24,26–30, 35–37].

Specific *KRAS* mutations induce different biological consequences by affecting differently the structural conformation and the function of the mutated protein [1,2,22]. In particular, CRC patient disease free survival and overall survival were shown to depend on the type of *KRAS* mutation [3,11]. In the latter study, in particular, it was shown that *KRAS* $G \rightarrow C$ and $G \rightarrow T$ transversions in CRC were related to a worse prognosis than G → A transitions. These findings are in general agreement with the literature data with a few exceptions [3,5].

An observation, however, that so far remained unexplained, is that *KRAS* G → A transitions in colorectal cancerized adenomas [16] and in adenocarcinomas [15] are more frequent than G → C/T transversions while in the precursor adenomas their incidences are about equal. More precisely, while *KRAS* G → C/T transversions range between 10 and 15% during the entire adenoma-carcinoma sequence, the G → A transitions frequency raises from about 10% in the adenomas to above 30% in the carcinomas.

The present study indicates that *KRAS* G → A transitions strongly orientate DNA diploid adenoma morphogenesis toward the villous architecture, that in turn is known to be a reliable predictor of higher risk of cancer and may justify the relative increase of *KRAS* G→ A transitions in CRC.

The study was conducted, after exclusion of serrated polyps [39] or polyps with sectors of serrated morphology, on 159 endoscopically removed and histologically proven colorectal polypoid adenomas (size range: 5–80 mm; median 15 mm). The patients were 137 (60% men and 40% women aged 33–86; median 65 years) with a negative family history for colorectal neoplasia. Twenty-seven percent of adenomas were located in the proximal colon, 73% in the distal colon. Polyps were divided in two parts by a central mid-sagittal section. One part was fixed in 10% buffered formalin, embedded in paraffin and stained with H&E and Feulgen stain. The other part was immediately frozen in liquid nitrogen to provide multiple samples for DNA flow cytometry-sorting and *KRAS* analysis using a H&E stained cryostat section as a histotopographic reference.

In accordance with WHO criteria [18], tubular adenomas were composed of branching neoplastic tubules in at least 80% of the tumour and villous adenomas were composed of leaf- or finger-like processes in at least 80% of the tumor; in tubulo-villous adenomas tubular and villous structures contributed to more than 20% of the tumour. Dysplasia was graded into low and high grade; the diagnostic grade was based on the most severely dysplastic area, independently on its extension.

DNA content flow cytometry was done with use of suspensions of nuclei stained with 4,6-diamidino-2-phenylindole-2-hydrochloride (DAPI, Sigma Chemical Co., St. Louis, MO). Four parameters were evaluated: nuclear DAPI fluorescence, proportional to DNA content, forward and perpendicular nuclear scatter signals, which reflect nuclear size and internal structure and proved to be useful to separate inflammatory from epithelial nuclei and DNA Index values (DI $= 1$ for diploidy and DI $\neq 1$ for aneuploidy) [12]. Sorting of nuclei, based on DNA and scatter, allowed to enrich for epithelial DNA diploid nuclei and to provide almost pure populations of epithelial nuclei among the DNA aneuploid subpopulations. Multiple aliquots of about 20,000 sorted nuclei were used for extraction of high molecular genomic DNA according to standard methods [32]. Specific DNA amplification by polymerase chain reaction was done to perform the analysis of *KRAS* mutations using an oligonucleotide 20-mers panel (TIB MOLBIOL, Advanced Biotechnology Center, Genoa, Italy), as previously described in details [13].

Data analysis was generated using SAS software (Copyright, SAS Institute Inc., Cary, NC, USA). Fisher exact test (in $2 \times 2$ tables) and Pearson's chi-square (in larger contingency tables) were used to evaluate the statistical significance of the relationships between two parameters. Cochran–Armitage Test for Trend was used to evaluate trends in contingency tables. Statistical tests were calculated two sided, and $p$-values $<0.05$ were considered statistically significant.

In the following we report the 2 contingency tables crossing the *KRAS* status of human sporadic colorectal adenomas (subdivided in wild type, G $\rightarrow$ A transitions and G $\rightarrow$ C/T transversions) with the DNA Index (DI) (in 116 cases) and with the adenoma architecture (categorized as tubular, tubulo-villous and villous) (in 159 cases). Both show highly statistically significant relationships among the investigated parameters (respectively $P = 0.004$ and $P < 0.0005$). While the G $\rightarrow$ C/T transversions were positively associated with DNA aneuploidy ($P = 0.003$ Fisher exact test), G $\rightarrow$ A transitions were associated with a dominant villous component (>80%) ($P = 0.001$ Fisher exact test) and prevalently occurred in DNA diploid adenomas. The DNA aneuploidy incidences in G $\rightarrow$ C/T and G $\rightarrow$ A mutated adenomas were respectively 61.5% and 18.8% while the *KRAS* wild type adenomas were aneuploid in 20% of the cases. Villous growth incidences among wt, G $\rightarrow$ C/T and G $\rightarrow$ A mutated adenomas were 37.5, 12.5 and 50%. When taking together wt and G $\rightarrow$ C/T cases in a $2 \times 3$ table, the frequencies of G $\rightarrow$ A transitions in tubular, tubulo-villous and villous adenomas were respectively 8, 19 and 50% ($P = 0.0002$, test for trend).

Moreover, within the subgroup of 88 DNA diploid adenomas in the present series, a dominant villous component was present in 5/75 cases (7%) with wild type *KRAS* and G $\rightarrow$ C/T transversions versus 6/13 cases (46%) with *KRAS* G $\rightarrow$ A transitions ($P = 0.002$ Fisher exact test).

It is well established that risk of cancerization progressively increases in large adenomas with respect to the small ones, and that adenomas with extensive villous architecture have a higher malignant potential than tubular adenomas (40% vs 5%) [23,40]. Conflicting results were also reported though disagreement could be partly ascribed to insufficient standardization and low reproducibility of histologic criteria guiding grading of dysplasia and evaluation of the histological type [38].

Little is known about the morphogenetic steps leading to the villous growth pattern in colorectal adenomas. *BRAF* and *KRAS* were already reported to be associated with villous morphology [41]. It is unlikely, however, that a single molecular event triggers and sustains the development of villi. For example, also somatic *APC* gene somatic mutations and villous type were shown to be associated [6].

In this scenario, our study provides a new refined analysis and suggests that not all *KRAS* mutations but mainly the *KRAS* G $\rightarrow$ A transitions are among the early events that are likely to confer to sporadic DNA diploid adenomas an increase risk of tumor progression through villous component expansion.

In the present series of cases, grade of dysplasia (even if strictly evaluated in terms of extension throughout the polyp) was independent from the type of growth and architecture and from the *KRAS* mutation spectrum. Similarly, size and *KRAS* status, that were addressed in previous studies [33], were found to be independent.

In a subnumber of 69 unselected cases of the present series, we preliminarily observed a statistically significant association ($P = 0.02$) of *KRAS* G $\rightarrow$ A transitions with an increased frequency of apoptosis according to the morphologic criteria of Leuchtenberger [31] which was not seen when we evaluated apoptosis according to the criteria of Kerr [19]. This observation will be further investigated with the aim to test if *KRAS* G $\rightarrow$ A transitions may lead to an imbalance of proliferation and apoptosis which accompanies villous growth.

Table 1

| KRAS status | DNA status | | Total | Histologic (architectural) subtype | | | Total |
|---|---|---|---|---|---|---|---|
| | Diploid DI = 0 | Aneuploid DI ≠ 0 | | Tubular | Tubulo-villous | Villous | |
| wt | 70 | 17 (20%) | 87 | 78 | 30 | 6 (37.5%) | 114 |
| G → C/T | 5 | 8 (62%) | 13 | 9 | 9 | 2 (12.5%) | 20 |
| G → A | 13 | 3 (19%) | 16 | 8 (8%) | 9 (19%) | 8 (50%) | 25 |
| Total | 88 | 28 | 116 | 95 | 48 | 16 | 159 |

In conclusion, the present data indicate that different types of *KRAS* mutations are linked with different types of progression and morphogenesis of colorectal polypoid adenomas. Whilst *KRAS* G → C/T transversion mutations appear early events related to a type of evolution associated with CIN and aneuploidy [5, 13,14], G → A transitions, mainly present in DNA diploid adenomas, appear linked with the expansion of a villous architecture of growth. The link of this type of growth with increased malignant potential and with *KRAS* G → A transitions appears to explain the observed selection of this type of *KRAS* mutations in CRC.

Mauro Risio[a], Davide Malacarne[b] and Walter Giaretti[b,*]

[a]*Department of Pathology, IRCC, Institute for Cancer Research and Treatment, Candiolo, Turin, Italy*

[b]*Department of Diagnostic Technologies, Lab. Biophysics-Cytometry, IST, National Institute for Cancer Research, Genoa, Italy*

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*Corresponding author. Fax: +39 010 5600711; E-mail:walter.giaretti@istge.it.

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