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Chromosomal instability, aneuploidy, and gene mutations in human sporadic colorectal adenomas

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Abstract. Whether *in vivo* specific gene mutations lead to chromosomal instability (CIN) and aneuploidy or viceversa is so far not proven. We hypothesized that aneuploidy among human sporadic colorectal adenomas and *KRAS2* and *APC* mutations were not independent. Additionally, we investigated if 1p34–36 deletions by dual target FISH were associated with aneuploidy. Among 116 adenomas, 29 were DNA aneuploid by flow cytometry (25%) and 29 were *KRAS2* mutated (25%). *KRAS2* mutations were associated with aneuploidy ($P = 0.02$). However, while G–C and G–T transversions were strongly associated with DNA aneuploidy ($P = 0.007$), G–A transitions were not. Within a second series of 61 adenomas, we found, instead, that *APC* mutational status and aneuploidy by flow cytometry were not associated. However, a statistically significant association was found with specific *APC* mutations, i.e., occurring in the mutation cluster region (MCR, codons 1200–1500) or downstream ($P = 0.016$). Finally, the correlation of 1p34–36 deletions with flow cytometric and FISH detected aneuploidy was also significant ($P = 0.01$). Specific *KRAS2* and *APC* mutations and loss of genes in the 1p34–36 region appear associated with aneuploidy suggesting that these events are not independent and may cooperate in inducing human sporadic colorectal adenomas. A cause effect relationship between gene mutations and aneuploidy remains, however, to be demonstrated.

Keywords: Aneuploidy, chromosomal instability, oncogenes, tumor suppressor genes

1. Introduction

Most human solid tumors show a plethora of recurrent numerical and structural chromosomal aberrations [13,19] (see also The Mitelman Database of Chromosome Aberrations in Cancer at http://cgap.nci.nih.gov/Chromosomes/Mitelman), which revealed distinct and converging pathways of karyotypic evolution by multivariate analyses [16]. Losses or gains of defined chromosomal regions or loss of heterozygosity (LOH) were observed in human sporadic colorectal adenomas of very small size (range 1–3 mm) [28]. These findings are in agreement with other studies performed with independent techniques such as G-banding karyotyping [2], DNA content flow cytometry [9], interphase fluorescence in situ hybridization (FISH) [3,6,14], and gain/loss of DNA by comparative genomic hybridization (CGH) [15,25], that allowed detection of both numerical and structural chromosome aberrations. Gains of chromosomes 7, 13 and 20, loss of chromosome 18, and deletion of 1p among human sporadic colorectal adenomas are well documented [2,3,14,17]. Among 70 cases examined in a study by Bomme et al. [3] using FISH with pericentromeric region probes, gains of chromosomes 7, 13 and 20 were present respectively at 34%, 44%, and 32%. The median proportion of cells with trisomy was larger than 50%. Parallel G-banding karyotyping of 64 cases was in good agreement with these data. In particular, trisomy 7 was detected in non-neoplastic cells [17] and was present alone in 5 adenomas [2], suggesting a pathogenetic role of trisomy 7 in colorectal tumorigenesis. FISH was also used by Herbergs and colleagues [14] to detect trisomy 7 as the most frequently occurring chromosome numerical aberration in sporadic colorectal adenomas (13/35 cases, 37%). Ried and collaborators [25] found trisomy 7 by CGH in 5/26 (19%) cases of sporadic colorectal adenomas, while the incidence of 7+ in the study of Hermsen et al. by CGH [15] was more than 40%, but limited to the adenomatous components of adenomas which progressed into early cancer. Several investiga-

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tions by G-banding and FISH reported rearrangements of chromosome 1 and, in particular, deletions of its short arm, as one of the most common early structural changes in colorectal adenomas [3,6]. Allelic imbalances at *APC* locus also represent an early event since they were observed in adenomas with an average size of 2 mm at a frequency of 55% [28].

Aneuploidy in colorectal adenomas was also extensively evaluated by DNA content flow cytometry which provides the degree of DNA aneuploidy or DNA Index (DI) and unique information on the presence of multiple DNA abnormal subpopulations or heteroploidy [9,10]. DI values were highly heterogenous and characterized by a non-random distribution with modal values at DI = 0.9, 1.15, 1.50, 1.80, 2.0 and 2.2. A valley was clearly evident at DI = 1.3–1.4 which separated near-diploid subpopulations from near-triploid ones. DNA aneuploid adenomas were characterized, in the 72% of cases, by DI values in the near-diploid region (DI $\leqslant$ 1.4), while, on the opposite, the majority of aneuploid adenocarcinomas (about 72%) were with DI $>$ 1.4.

KRAS2 and *APC* mutations were also commonly observed in colorectal adenomas and in their hypothetical precursor non-dysplastic and dysplastic aberrant crypt foci [30]. Specific *KRAS2* mutations and DNA aneuploidy were reported to be associated in human colorectal adenomas [10,11] and in cell lines [23,24,26]. That the RAS pathway and spindle assembly may collide in yeast was proposed [27].

APC mutations, on the other hand, would have a direct role in CIN and subsequent karyotypic abnormalities [8,18]. According to experiments using mouse embryonic stem cells carrying *APC* mutated alleles, APC truncated proteins, which miss the carboxyl-terminal sequences, would loose their interaction with the elongating spindle microtubules and the kinetochores, respectively via hBUB1 and EB1, and generate aneuploidy. Mutations in *hBUB1* were also implicated in CIN, but were rarely found in colorectal cancer [5,20].

2. Aneuploidy and *KRAS2* mutations

Among 116 examined human sporadic colorectal adenomas, 29 were aneuploid (25%) and 29 were *KRAS2* mutated (25%). *KRAS2* mutations were analyzed using sorted epithelia nuclei as previously described [10]. *KRAS2* G–C and G–T transversions, but not G–A transitions in codons 12 and 13 of exon 1, were strongly associated with DNA aneuploidy by logistic regression analysis which provided an Odd Ratio = 6.1 [11] and by contingency table analysis (Table 1). DNA aneuploidy was also detected in limiting dilution experiments after about twenty doubling times for murine cells transfected with the human G–C *KRAS2* mutated oncogene [23] in association with

Table 1

KRAS2 and *APC* mutations and deletions at 1p36 versus DNA Index among human sporadic colorectal adenomas

	DI = 1	DI $\neq$ 1	P
KRAS2			
wild type	70 (80%)	17 (20%)	
mutated	17 (59%)	12 (41%)	$P_{12} = 0.02$
G $\rightarrow$ A transitions	12 (70%)	5 (30%)	$P_{13} = 0.35$
G $\rightarrow$ C/T transversions	5 (42%)	7 (58%)	$P_{14} = 0.007$
APC			
wild type	30 (71%)	12 (29%)	
mutated	10 (53%)	9 (47%)	$P_{12} = 0.24$
<MCR	6 (86%)	1 (14%)	$P_{13} = 0.66$
$\geqslant$MCR	4 (33%)	8 (67%)	$P_{14} = 0.04$
			$P_{134} = 0.024$
			$P_{(1+3)4} = 0.016$
1p36 deletions			
absent	18 (86%)	3 (14%)	
present	1 (20%)	4 (80%)	$P_{12} = 0.01$

DI, DNA Index; P, P-values obtained by the Fisher's exact test; MCR, mutation cluster region (codons 1200–1500).

inhibition of apoptosis and loss of check-points in the G2M cell cycle phases [24]. New experiments with cell lines derived from human colorectal adenomas and carcinomas and permanently transfected with specific *KRAS2* G–C and G–T transversions and G–A transitions are in progress.

3. Aneuploidy and *APC* mutations

A role of APC in the origin of CIN and aneuploidy in an *in vitro* model was suggested [8,18]. We aimed to verify this hypothesis *in vivo* among human sporadic colorectal adenomas. Aneuploidy was associated with an abnormal nuclear DNA Index (DI $\neq$ 1) as evaluated by flow cytometry. With this technique we also sorted epithelial nuclei, as previously detailed [10], in which analysis of *APC* mutation spectrum by DNA sequencing was performed. Amongst 61 adenomas, 33% exhibited aneuploidy and 31% *APC* mutations. Microsatellite instability, investigated in a subset of 15 adenomas, was present in 1 case. Among the examined 14 *APC* mutated adenomas, LOH was detected in 4 cases and a double *APC* mutation in 1 case. The incidence of aneuploidy among *APC* wild type and mutated adenomas was respectively 29% and 47% ($P = 0.24$), suggesting that *APC* mutational status and aneuploidy were not associated (Table 1). Of the 7 *APC* mutations occurring upstream MCR, 6 were associated with diploid adenomas. Of the 12 mutations occurring in the MCR or downstream, 8 were associated with aneuploid adenomas (67%). A statistically significant association between *APC* mutation type and aneuploidy was observed (Table 1), suggesting that the specific *APC* mutations within and downstream MCR may be associated with aneuploidy.

4. Aneuploidy and 1p deletions

We investigated the numerical aberrations of chromosomes 1, 7, 17, 18, the 1p deletions and the nuclear DNA content as obtained by flow cytometry, in a series of 34 human sporadic colorectal adenomas. From these adenomas, 51 intra-adenoma regions were microdissected according to two degrees of dysplasia and presence of foci of early cancer. Isolated epithelial nuclei were analyzed by FISH using centromeric probes for chromosomes 7, 17 and 18 and, in a double-target analysis, a centromeric probe for chromosome 1 simultaneously with a telomeric probe mapping to the 1p36 band [6]. Considering the presence of numerical aberrations for at least one among the investigated chromosomes and/or abnormal DNA content, aneuploidy incidence was 35%, while 1p deletion incidence was 38%. The correlation of 1p deletions, mainly at 1p36, with aneuploidy was highly significant (Table 1), suggesting that loss of genes in this region may be implicated in CIN *in vivo*.

5. Conclusion

The significance of aneuploidy in cancer and the knowledge on the mechanisms causing CIN and aneuploidy still remain very limited.

In the present study, based on our previous investigations among human sporadic colorectal adenomas [10,11], we report further evidence for the association of *KRAS2* G–T/C transversions, but not G–A transitions, with DNA near-diploid aneuploidy, suggesting a possible involvement of specific *KRAS2* mutations in CIN *in vivo*. A link of *KRAS2* mutations with aneuploidy *in vitro* was also shown using a mouse cell line transfected with a *KRAS2* G–C transversion [23]. Similar results were also obtained using human transfected cell lines [26]. The mechanisms of KRAS2-mediated CIN and aneuploidy, however, are still not well understood. A study using mouse cells suggests the importance of G2M checkpoints and inhibition of apoptosis [24]. Other observations in yeast suggest the interaction of RAS-dependent specific proteins with the cytoskeleton and the mitotic spindle [27].

Using a limited series of 61 human sporadic colorectal adenomas, we also reported that *APC* mutations *in vivo* were not significantly associated with aneuploidy. However, subset group analysis, so far limited to small sample sizes, suggested that the specific *APC* mutations occurring within and downstream MCR might be associated with aneuploidy and have eventually a role in CIN *in vivo.* This last observation would be partly in agreement with previous studies using mouse embryonic stem cells carrying *APC* mutated alleles, suggesting that *APC* mutational status could be directly linked with CIN and aneuploidy [8,18].

Additionally, based on previous investigations, we also reported that 1p34–36 deletions were strongly associated with aneuploidy, suggesting that loss of genes in this region may be implicated in CIN *in vivo*. No gene level investigations are so far available linking gene mutations in this chromosomal region with aneuploidy [7,29].

Many studies are presently conducted to attempt a better understanding of the mechanisms causing CIN and aneuploidy. An interesting view is that aneuploidy, proposed to be a primary cause of cancer, is due to an abnormal dosage of normal genes [21]. Alternatively, in a recent comprehensive review, more than 70 genes have to date been reported that monitor genome integrity and CIN and coordinate cell cycle progression with DNA repair [1]. Among these, p53 inactivation in association with the dysfunction of telomeres was suggested as one of the most important driving forces of CIN [22]. Unfortunately, inactivation of p53 is quite rare in colorectal adenomas with moderate dysplasia while aneuploidy is already quite common. Additionally, other studies failed to prove the involvement of p53 in CIN both *in vitro* [4] and *in vivo* [12]. Other CIN driving mechanisms may include microtubule dynamic instability, kinetochore structure and function, chromosome condensation and sister-chromatid cohesion [5,20]. Cell cycle checkpoints and apoptosis were also postulated to play a role in CIN, though the relative importance of the various mechanisms is so far unknown.

The understanding of CIN mechanisms in association with specific gene mutations need additional work with the use of *in vitro* and *in vivo* models. Whether specific gene mutations lead to aneuploidy or viceversa is so far not proven, and the hypothesis that specific carcinogens in the human large intestine induce aneuploidy in parallel with specific gene mutations cannot be ruled out. It is likely that both subtle gene mutations and large scale chromosomal alterations cooperate to tumor genesis and progression in an evolutionary process characterized by divergence factors generating heterogeneity and convergence factors generating selection. An initial altered gene expression state, due to an abnormal dosage of normal and mutated genes, may lead to an equilibrium gene expression state which represents a specific tumor phenotype.

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