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Cytoplasmic localization of p27 (CDKN1B / KIP1) in colorectal cancer: inverse correlations with nuclear p27 loss, microsatellite instability and CpG island methylator phenotype (CIMP)

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Abstract

Cytoplasmic mislocalization of p27 (cyclin-dependent kinase inhibitor-1B, CDKN1B/KIP1) is caused by activated AKT1, and has been associated with poor prognosis in various cancers. CpG island methylator phenotype (CIMP) in colorectal cancer is characterized by extensive promoter methylation, and is associated with microsatellite instability-high (MSI-H) and *BRAF* mutations. We have recently shown a positive correlation between MSI/CIMP and loss of nuclear p27. However, no study has examined cytoplasmic p27 mislocalization in relation to CIMP and MSI in colorectal cancer. Using MethyLight assays, we quantified DNA methylation in eight CIMP-specific gene promoters {*CACNA1G*, *CDKN2A* (p16), *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*} in 853 colorectal cancer samples obtained from two large prospective cohorts. We assessed expressions of nuclear and cytoplasmic p27 and nuclear p53 by immunohistochemistry. Cytoplasmic p27 expression was inversely associated with loss of nuclear p27 ($p<0.0001$), CIMP-high ($p<0.0001$), MSI-H ($p<0.0001$) and *BRAF* mutations ($p<0.0001$). The inverse association of cytoplasmic p27 with CIMP-high (or MSI-H) was independent of MSI (or CIMP) status. In addition, the inverse association of cytoplasmic p27 with CIMP-high was independent of *KRAS*/*BRAF* status. *BRAF* and *CDKN2A* (p16) methylation were not correlated with cytoplasmic p27 after stratification by CIMP status. The inverse associations of cytoplasmic p27 with MSI-H and CIMP-high were much more pronounced in p53-negative tumors than p53-positive tumors. In conclusion, cytoplasmic p27 expression is inversely associated with MSI-H and CIMP-high, particularly in p53-negative tumors, suggesting interplay of functional losses of p27 and p53 in the development of various molecular subtypes of colorectal cancer.

Keywords

colon cancer; CIMP; p27; cyclin-dependent kinase inhibitor; MSI

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Introduction

Progression through the cell cycle involves sequential activation and inactivation of cyclin-dependent kinases (CDKs) [1]. CDKs are activated through association with positive regulators (cyclins) and inactivated by cyclin-CDK inhibitors. p27 {*CDKN1B* (cyclin-dependent kinase inhibitor 1B) / KIP1} is one of the cyclin-CDK inhibitors and plays a key role in preventing progression into S phase of the cell cycle [1]. Regulation of p27 levels is achieved post-translationally through ubiquitin-mediated protein degradation [2]. The F-box protein SKP2 has been identified as the substrate recognition component that binds and targets p27 for ubiquitination and subsequent degradation [3]. Low levels of p27 have been associated with tumor progression and poor prognosis in various cancers including colon cancer [4,5]. Although mutations or deletions of p27 allele rarely occur, down-regulation of p27 in colorectal cancer mainly result from abnormal activation of ubiquitin-mediated proteolysis [4,5]. *CDKN1B* (p27) promoter has not been shown to be methylated in colorectal cancer [6]. In addition to loss of nuclear p27 expression, cytoplasmic mislocalization of p27 has been observed in colon cancer [7]. In various cancers, cytoplasmic p27 mislocalization has been associated with activated AKT1 (protein kinase B) [8,9], overexpression of cyclin D3 [10], and poor prognosis [1,11]. However, biological implications and differences between p27 loss and p27 mislocalization in colorectal cancer, particularly in relation to other molecular alterations, have not been comprehensively evaluated.

Transcriptional inactivation by cytosine methylation at promoter CpG islands of tumor suppressor genes is thought to be an important mechanism in human carcinogenesis [12]. A number of tumor suppressor genes are silenced by promoter methylation in colorectal cancer [12]. Promoter CpG island methylation has been shown to occur early in colorectal carcinogenesis [13]. A subset of colorectal cancers exhibit promoter methylation in multiple genes, referred to as the CpG island methylator phenotype (CIMP) [12,14]. CIMP-positive colorectal tumors appear to have a distinct clinical, pathologic and molecular profile, including associations with female sex, proximal tumor location, mucinous and poor differentiations, microsatellite instability (MSI) and *BRAF* mutations [14–17]. We have recently demonstrated that both MSI and CIMP are correlated positively with loss of nuclear p27 [18], and inversely with down-regulation of p21 (CDKN1B / KIP1) [19], another cyclin-dependent kinase inhibitor. However, no study to date has examined relationship between cytoplasmic p27 expression, MSI and CIMP in colorectal cancer. In this study, using quantitative real-time PCR (MethyLight) assays [16,20,21], and relatively unbiased samples of colorectal cancer from two large prospective cohort studies, we show that loss of p27 expression and cytoplasmic p27 localization have opposite molecular features in terms of MSI and CIMP. MethyLight assays can reliably distinguish high from low levels of DNA methylation, the latter of which likely have little or no biological significance [22].

Materials and methods

Study group

We utilized the databases of two large prospective cohort studies; the Nurses' Health Study (N = 121,700 women followed since 1976) [23], and the Health Professional Follow-up Study (N = 51,500 men followed since 1986) [24]. Informed consent was obtained from all participants prior to inclusion in the cohorts. All cohort participants were free of cancer at the time of study entry. A subset of the cohort participants developed colorectal cancers during prospective follow-up. We included cases only if there was adequate paraffin-embedded tumor tissue and results were available for MSI status and p27 at the time of this study. As a result, a total of 855 colorectal cancer cases (364 from the men's cohort and 491 from the women's cohort) were included in this study. Many tumors have been previously characterized for status of CIMP, MSI, *KRAS*, *BRAF* and nuclear p27 [18,19,25]. However, no tumor has previously

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been studied for cytoplasmic p27 expression. Tissue collection and analyses were approved by the Institutional Review Boards.

Microsatellite instability (MSI) analysis

Genomic DNA was extracted as previously described [16]. Whole genome amplification (WGA) of genomic DNA was performed by PCR using random 15-mer primers [26]. Methods for MSI analysis were previously described [16]. In addition to D2S123, D5S346, D17S250, BAT25 and BAT26 (the NCI panel), we used BAT40, D18S55, D18S56, D18S67 and D18S487 (i.e., a 10-marker panel). "MSI-high (MSI-H)" was defined as instability in 30% or more of the markers, "MSI-low (MSI-L)" as instability in less than 30% of the markers, and "microsatellite stability (MSS)" as no unstable marker.

Real-time PCR (MethyLight) for quantitative DNA methylation analysis

Sodium bisulfite treatment on genomic DNA was performed as previously described [22]. For DNA methylation analysis, we typically used 1 to 2 tissue sections (10 μm thick) when large tumor sections were available. Real-time PCR to measure DNA methylation (MethyLight) was performed as previously described [20,21,27]. We used ABI 7300 (Applied Biosystems, Foster City, CA USA) for quantitative real-time PCR. Using nine sets of primers and probes, we amplified eight CIMP-specific promoters (*CACNA1G*, *CDKN2A* (*p16/INK4A*), *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*) [16,17] and *COL2A1*. The use of these eight markers in a CIMP-specific panel has been validated by examining 920 colorectal cancers, and all of the eight markers showed good sensitivity and specificity for the prediction of overall CIMP status (manuscript submitted). *COL2A1* (the collagen 2A1 gene) was used to normalize for the amount of input bisulfite-converted DNA [21,22]. Primers and probes were previously described as follows: *CACNA1G*, *CRABP1* and *NEUROG1* [16,17]; *CDKN2A* and *COL2A1* [21]; *MLH1* [22]; and *IGF2*, *RUNX3* and *SOCS1* [17]. The percentage of methylated reference (PMR, i.e., degree of methylation) at a specific locus was calculated by dividing the *GENE:COL2A1* ratio of template amounts in a sample by the *GENE:COL2A1* ratio of template amounts in *Sss*I-treated human genomic DNA (presumably fully methylated) and multiplying this value by 100 [28]. A PMR cutoff value of 4 was based on previously validated data [21, 22,28]. Based on the distribution of PMR values at the *CRABP1* and *IGF2* loci, we raised PMR cutoff to 6 for *CRABP1* and *IGF2*. Precision and performance characteristics of bisulfite conversion and subsequent MethyLight assays have been previously evaluated and the assays have been validated [22].

The CpG island methylator phenotype-high (**CIMP-high**) was defined as the presence of ≥6/8 methylated promoters, **CIMP-low** as the presence of 1/8 to 5/8 methylated promoters **CIMP-0** as the absence (0/8) of methylated promoters, based on the fact that CIMP-high and CIMP-low are associated with high *BRAF* and *KRAS* mutation rates, respectively [25].

Sequencing of *KRAS* and *BRAF*

Methods of *KRAS* and *BRAF* Pyrosequencing have been previously described.[25,26] Pyrosequecing was performed using the PSQ96 HS System (Biotage AB and Biosystems, Uppsala, Sweden) according to the manufacturer's instructions.

Tissue microarray (TMA) and immunohistochemistry for p27 and p53

Methods for p27 immunohistochemistry were previously described [29]. The extent of nuclear and cytoplasmic p27 expressions were visually estimated using whole tissue sections. Nuclear p27 expression was interpreted as "loss" (no staining, only weakly staining, or <10% of tumor cells positive for moderate/strong staining) (Figure 1A), positive in 10–49% of cells (1+), or positive in ≥ 50% of cells (2+) (Figure 1B). Cytoplasmic p27 expression was interpreted as

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negative (no staining or <10% of tumor cells staining) or positive (≥ 10% of tumor cells staining). Inflammatory cells and normal colonic epithelial cells served as internal positive controls.

Tissue microarrays (TMAs) were constructed for p53 analysis as previously described [30], using the Automated Arrayer (Beecher Instruments, Sun Prairie, WI USA). For p53 immunohistochemistry, deparaffinized tissue sections in a citrate buffer (BioGenex, San Ramon, CA) were microwaved in a pressure cooker at high power for 15 min. Tissue sections were incubated with 3% H_2O_2 (10 min) to block endogenous peroxidase, and then incubated with protein block (Vector Laboratories, Burlingame, CA) (10 min). Primary anti-p53 mouse monoclonal antibody (clone DO-1, Calbiochem, San Diego, CA) (dilution 1:50) was applied for 30 min at room temperature. Then, biotinylated secondary Multi-Link antibody (Biogenex) was applied (20 min), horse radish peroxidase avidin complex (Biogenex) was added (20 min) and sections were visualized by DAB (5 min) and methyl-green counterstain. We visually estimated the fraction of tumor cells with unequivocal strong nuclear staining for p53, by examining at least two tissue cores in TMAs, or the whole tissue section in each case for which there was not enough tissue for TMAs or results were equivocal in TMAs. p53 positivity was defined as 50% or more of tumor cells with unequivocal strong nuclear staining.

Appropriate positive and negative controls were included in each run of p27 and p53 immunohistochemistry. All immunohistochemically-stained slides were interpreted by a pathologist (S.O.) blinded from any other laboratory data.

Statistical analysis

In statistical analysis, chi-square test (or Fisher's exact test when any category was less than 10) was performed for categorical data, using the SAS program (version 9.1, SAS Institute, Cary, NC). All p values were two-sided, and statistical significance was set at $p \leq 0.05$.

RESULTS

Cytoplasmic p27 Localization Is Inversely Correlated with Loss of Nuclear p27

We examined nuclear and cytoplasmic p27 expressions in 855 colorectal cancer specimens by immunohistochemistry; 408 (48%) and 447 (52%) were positive and negative, respectively, for cytoplasmic p27 expression. Among the 855 tumors, 160 (19%) were extensively positive (2+) for nuclear p27, 427 (50%) were focally positive (1+) for nuclear p27, and the remaining 268 (31%) showed loss of nuclear p27. Figure 2 illustrates relationship between cytoplasmic and nuclear p27 expressions. Cytoplasmic p27 positivity was significantly more frequent in tumors with nuclear p27 1+ or 2+ expression (63–64%, $p < 0.0001$) than in tumors with nuclear p27 loss (12%), raising the possibility that cytoplasmic sequestration of p27 and loss of nuclear p27 expression tend to be mutually exclusive events in colorectal cancer. We used inflammatory cells and normal colonic epithelial cells as internal positive controls. Therefore, it is unlikely that this positive correlation was due to unstainable tumors with no or low overall antigenicity.

Cytoplasmic p27 expression is inversely associated with microsatellite instability (MSI) and CpG island methylator phenotype (CIMP)

We quantified DNA methylation in a panel of the eight CIMP-specific promoters (*CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*) [16,17] by MethyLight to determine CIMP status in tumors. The selection and use of these eight markers for the determination of CIMP status have been validated [16,17]. Among 833 tumors with DNA methylation data, 132 tumors (16%) were classified as CIMP-high (≥ 6/8 methylated promoters).

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Tumors with cytoplasmic p27 expression showed significantly lower frequencies of MSI-H (5.1%, $p < 0.0001$) and CIMP-high (6.6%, $p < 0.0001$) than tumors negative for cytoplasmic p27 (MSI-H 24%, and CIMP-high 24%) (Table 1). Among cytoplasmic p27-negative tumors, nuclear p27-negativity was associated with higher frequencies of MSI-H (29%, $p = 0.001$) and CIMP-high (30%, $p < 0.0001$) compared to nuclear p27 1+ positivity (MSI-H 15%, and CIMP-high 13%) (Table 1). Interestingly, among cytoplasmic p27-negative tumors, nuclear p27 2+ positive tumors also showed higher frequencies of MSI-H (25%, $p = 0.10$) and CIMP-high (31%, $p = 0.002$) than nuclear p27 1+ tumors (MSI-H 15%, and CIMP-high 13%). In contrast, among cytoplasmic p27-positive tumors, there were no significant differences in the MSI-H or CIMP-high frequencies between nuclear p27-negative, 1+ positive and 2+ positive tumors (data not shown).

In order to determine whether the inverse association of cytoplasmic p27 positivity with CIMP-high was due to a correlation between p27 and methylation of *CDKN2A* (p16, another important cell cycle regulator), we examined the frequencies of cytoplasmic p27 positivity among CIMP-high and CIMP-low/0 tumors stratified by *CDKN2A* methylation status (Supplemental Material 1, after Figure 5). For this analysis, *CDKN2A* was excluded from the CIMP panel to avoid its confounding effect on diagnosing CIMP status, and CIMP-high was defined as the presence of ≥5/7 methylated promoters. After stratification by CIMP status, *CDKN2A* methylation was not correlated with cytoplasmic p27 expression. In contrast, the inverse association between cytoplasmic p27 and CIMP-high was still present even after tumors were stratified by *CDKN2A* methylation status.

Cytoplasmic/nuclear p27 expression, and mutations of *KRAS* and *BRAF*

We examined relations between cytoplasmic/nuclear p27 expression and mutations of *KRAS* and *BRAF*. Tumors with cytoplasmic p27 expression showed a significantly lower frequency of *BRAF* mutations (6.6%, $p < 0.0001$) than tumors negative for cytoplasmic p27 (20%) (Table 2). Among cytoplasmic p27-negative tumors, nuclear p27-negativity was associated with a higher frequency of *BRAF* mutations (24%, $p = 0.04$) compared to nuclear p27 1+ positivity (*BRAF* mutations 15%). Cytoplasmic/nuclear p27 expression was not correlated with *KRAS* mutations.

Cytoplasmic p27 expression in various CIMP/*KRAS*/*BRAF* subtypes of colorectal cancer

In order to examine an association between cytoplasmic p27 and *KRAS/BRAF* mutations independent of CIMP status, we examined the frequencies of cytoplasmic p27 expression in six subtypes of colorectal cancer according to combined status of CIMP/*KRAS/BRAF* (Figure 3). There was no significant difference in the frequencies of cytoplasmic p27 expression between *BRAF*-mutated (*KRAS* wild-type) tumors and *BRAF/KRAS* wild-type tumors after tumors were stratified by CIMP status. Thus, *BRAF* mutations did not seem to be directly associated with cytoplasmic p27 expression.

Cytoplasmic p27 Expression in various MSI/CIMP subtypes of colorectal cancer

In order to examine an association between cytoplasmic p27 expression and CIMP-high (or MSI-H) independent of MSI (or CIMP) status, we examined the frequencies of cytoplasmic p27 expression in four subtypes of colorectal cancer stratified by both MSI and CIMP status (Figure 4). Overall, both MSI-H and CIMP-high appeared to be independently correlated (inversely) with cytoplasmic p27 expression. Among MSI-H tumors, MSI-H CIMP-high tumors showed a significantly lower frequency of cytoplasmic p27 expression (9.1%) than MSI-H CIMP-low/0 tumors (32%, $p = 0.002$). Among MSI-L/MSS tumors, MSI-L/MSS CIMP-high tumors showed a lower frequency of cytoplasmic p27 expression (39%) than MSI-L/MSS CIMP-low/0 tumors (52%, $p = 0.09$) though statistical significance was not reached.

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Thus, cytoplasmic p27 expression appeared to be inversely associated with CIMP-high independent of MSI status.

We also stratified tumors by CIMP status. Among CIMP-high tumors, MSI-H CIMP-high tumors showed a significantly lower frequency of cytoplasmic p27 expression (9.1%) than MSI-L/MSS CIMP-high tumors (39%, $p < 0.0001$). Among CIMP-low/0 tumors, MSI-H CIMP-low/0 tumors showed a lower frequency of cytoplasmic p27 expression (32%) than MSI-L/MSS CIMP-low/0 tumors (52%, $p = 0.02$). Therefore, cytoplasmic p27 expression was inversely associated with MSI-H independent of CIMP status.

Combined p27/p53 status, CIMP and MSI

Because of important roles of both p27 and p53 in regulating the cell cycle, we correlated combined status of p27 and p53 with CIMP and MSI. The inverse associations of cytoplasmic p27 expression with CIMP-high and MSI-H were much more pronounced among p53-negative tumors than p53-positive tumors (Figure 5, raw data in Supplemental material 2). For instance, p53-negative/cytoplasmic p27-negative tumors showed much higher frequencies of MSI-H (32%) and CIMP-high (31%) than p53-negative/cytoplasmic p27-positive tumors (MSI-H 7.2% and CIMP-high 5.6%) (Figure 5). These results suggest that the functional status of p27 is much more relevant in tumors with wild-type p53.

DISCUSSION

We conducted this study to examine significance of cytoplasmic localization of p27. We evaluated relationship between nuclear and cytoplasmic expression of p27 and other molecular features of colorectal cancer, including CpG island methylator phenotype (CIMP), microsatellite instability (MSI) and mutations in the *KRAS* and *BRAF* oncogenes. In the current study, the use of quantitative DNA methylation assays (MethyLight) as well as population-based samples of colorectal cancer from two large prospective cohorts has enabled us to precisely estimate the frequency of colorectal cancers with specific molecular features (such as CIMP-high, MSI-H, cytoplasmic p27 positive, etc.) at a population level. We have demonstrated that cytoplasmic p27 expression is inversely correlated with CIMP-high and MSI-high (MSI-H) in colorectal cancer. In addition, we have shown that cytoplasmic p27 is correlated with nuclear p27 expression (retention), raising the possibility that cytoplasmic sequestration of p27 and loss of nuclear p27 tend to be mutually exclusive events in colorectal carcinogenesis. Taken together with our previous data of positive correlations for nuclear p27 loss with MSI-H and CIMP-high in colorectal cancer [18], our current data imply that MSI-H CIMP-high tumors preferentially lose nuclear p27 whereas non-MSI-H non-CIMP-high tumors preferentially sequester p27 in cytoplasm.

Because CIMP-high and MSI-H (as well as CIMP-high and *BRAF* mutations) are tightly associated with each other [15–17], we stratified tumors according to CIMP and MSI status, and according to CIMP and *KRAS/BRAF* gene status. The inverse association of cytoplasmic p27 expression with CIMP-high (or MSI-H) appeared to be independent of MSI (or CIMP) status. In addition, the inverse association of cytoplasmic p27 expression with CIMP-high also appeared to be independent of *BRAF* status. However, the inverse correlation of cytoplasmic p27 expression with *BRAF* mutations failed to persist when tumors were stratified by both CIMP and *KRAS/BRAF* status. Thus, we conclude that cytoplasmic p27 expression is inversely associated with CIMP-high and MSI-H, but not directly with *BRAF* mutations.

Because of important roles of both p27 and p53 in regulating the cell cycle, we examined whether p53 status in colorectal cancer might modify molecular characteristics of tumors with various cytoplasmic/nuclear p27 status. Interestingly, the inverse associations of cytoplasmic p27 expression with CIMP-high and MSI-H were much more pronounced among p53-negative

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(mostly wild-type) tumors than p53-positive (mostly mutated) tumors. Our results imply that functional status of p27 may be much more important in p53 wild-type tumors than p53-mutated tumors. Although p53 immunohistochemistry has been shown to have both false positives and false negatives for the assessment of *TP53* gene mutations, when higher threshold of p53 positivity is used (as in the current study), p53 immunohistochemistry can generally predict the presence or absence of mutations of *TP53* [31].

A cause and biological significance of cytoplasmic mislocalization of p27 in cancer have been investigated. Liang et al. [8] have demonstrated that activated AKT1 (protein kinase B) phosphorylates p27, impairing nuclear import of p27 in breast cancer cells, and that cytoplasmic mislocalization of p27 is associated with poor prognosis in breast cancer. An association between cytoplasmic p27 mislocalization and phosphorylation of AKT1 has also been observed in acute myelogenous leukemia [9] and thyroid carcinoma [32]. Cytoplasmic mislocalization of p27 has been shown to be associated with poor prognosis in various cancers [1,11]. Cytoplasmic mislocalization of p27 has also been reported in colon cancer [7]; however, its biological significance in colon cancer has not been studied.

The prognostic significance of nuclear p27 loss in colorectal cancer has been examined in previous studies, which have shown that nuclear p27 loss was a significant predictor of worse survival by multivariate analysis [4,33,34]. Manne et al. [35] examined p27 expression in colorectal cancer stratified by stage and found that nuclear p27 loss was associated with local recurrence and poor survival only for stage III colorectal cancer. The authors also found that p27 loss was associated with poor differentiation (in stage II tumors) and distal location [35]. In contrast, Palmqvist et al. [33] showed that p27 loss was associated with proximal location. These discrepant results may be attributable to differences in patient populations and criteria of p27 interpretation. We have previously examined nuclear p27 expression in relation to response to combination chemotherapy against advanced colorectal cancer, and shown that p27 positivity might confer a better response rate although statistical significance was not reached [29].

In conclusion, cytoplasmic localization of p27 in colorectal cancer is inversely associated with nuclear p27 loss, CIMP-high and MSI-H. Our results imply that there are mutually exclusive pathways of functional inactivation of p27, namely loss of nuclear p27 and cytoplasmic sequestration. Exact biological significance of cytoplasmic p27 mislocalization and influence of CIMP and MSI status on biological effect of p27 need to be further elucidated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations and HUGO Gene Nomenclature Committee (HGNC)-approved official gene symbols used

CACNA1G
: calcium channel, voltage-dependent, T type alpha-1G subunit

CDKN1B
: cyclin-dependent kinase inhibitor 1B (p27/KIP1)

CDKN2A
: cyclin-dependent kinase inhibitor 2A (p16/INK4A)

CIMP
: CpG island methylator phenotype

CRABP1
: cellular retinoic acid binding protein 1

IGF2
: insulin-like growth factor 2

MSI

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microsatellite instability

MSI-H
microsatellite instability-high

MSI-L
microsatellite instability-low

MSS
microsatellite stable

NEUROG1
neurogenin 1

PMR
percentage of methylated reference (degree of DNA methylation)

RUNX3
runt-related transcription factor 3

SOCS1
suppressor of cytokine signaling 1

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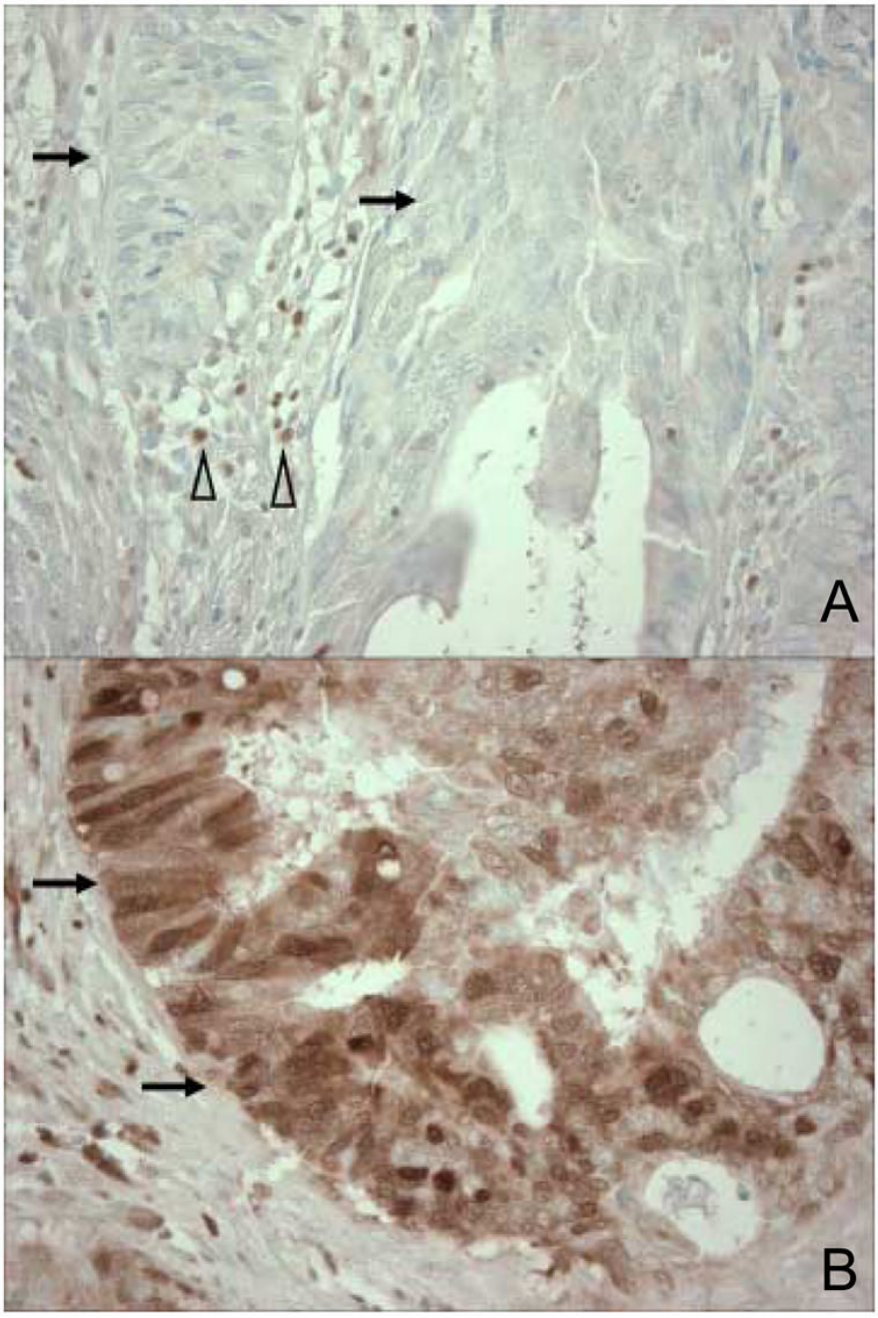

Figure 1. Cytoplasmic or nuclear p27 expression in colorectal cancer

A. Tumor cells with no cytoplasmic or nuclear p27 expression are indicated by arrows. Inflammatory cells serve as internal positive controls (empty arrowheads). B. Tumor cells with cytoplasmic and nuclear p27 expression are indicated by arrows.

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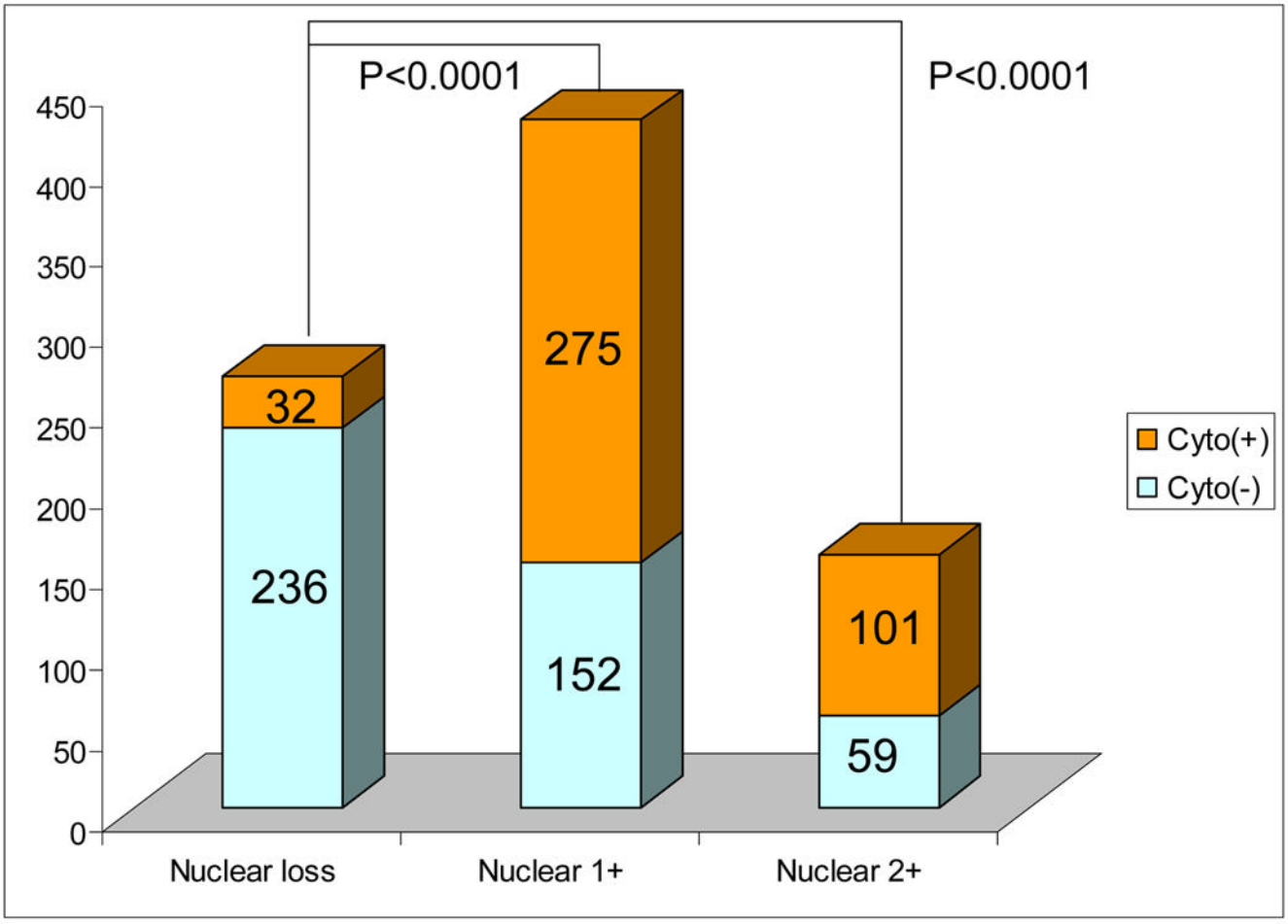

Figure 2. Relationship between nuclear and cytoplasmic expressions of p27 in 855 colorectal cancers

The Y axis indicates the number of tumors. P values indicate statistical significance levels when comparing the fractions of tumors expressing cytoplasmic p27. Cyto(+), cytoplasmic p27 positive; Cyto(−), cytoplasmic p27 negative.

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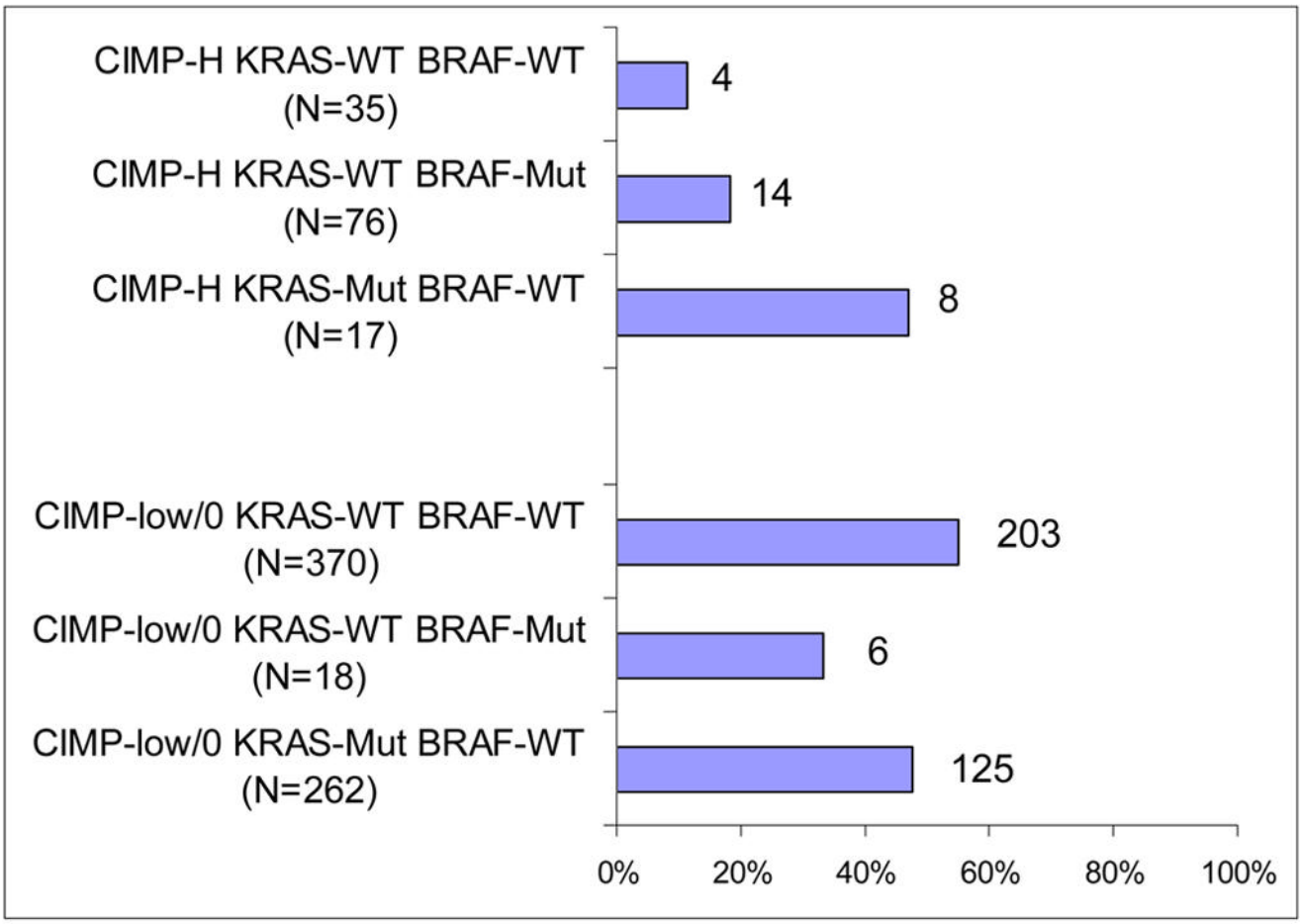

Figure 3. Frequency of cytoplasmic p27+ in various CIMP/KRAS/BRAF subtypes of colorectal cancer

BRAF or *KRAS* mutations do not appear to be consistently correlated with cytoplasmic p27 expression after stratification by CIMP status. CIMP, CpG island methylator phenotype; Mut, mutated; WT, wild-type.

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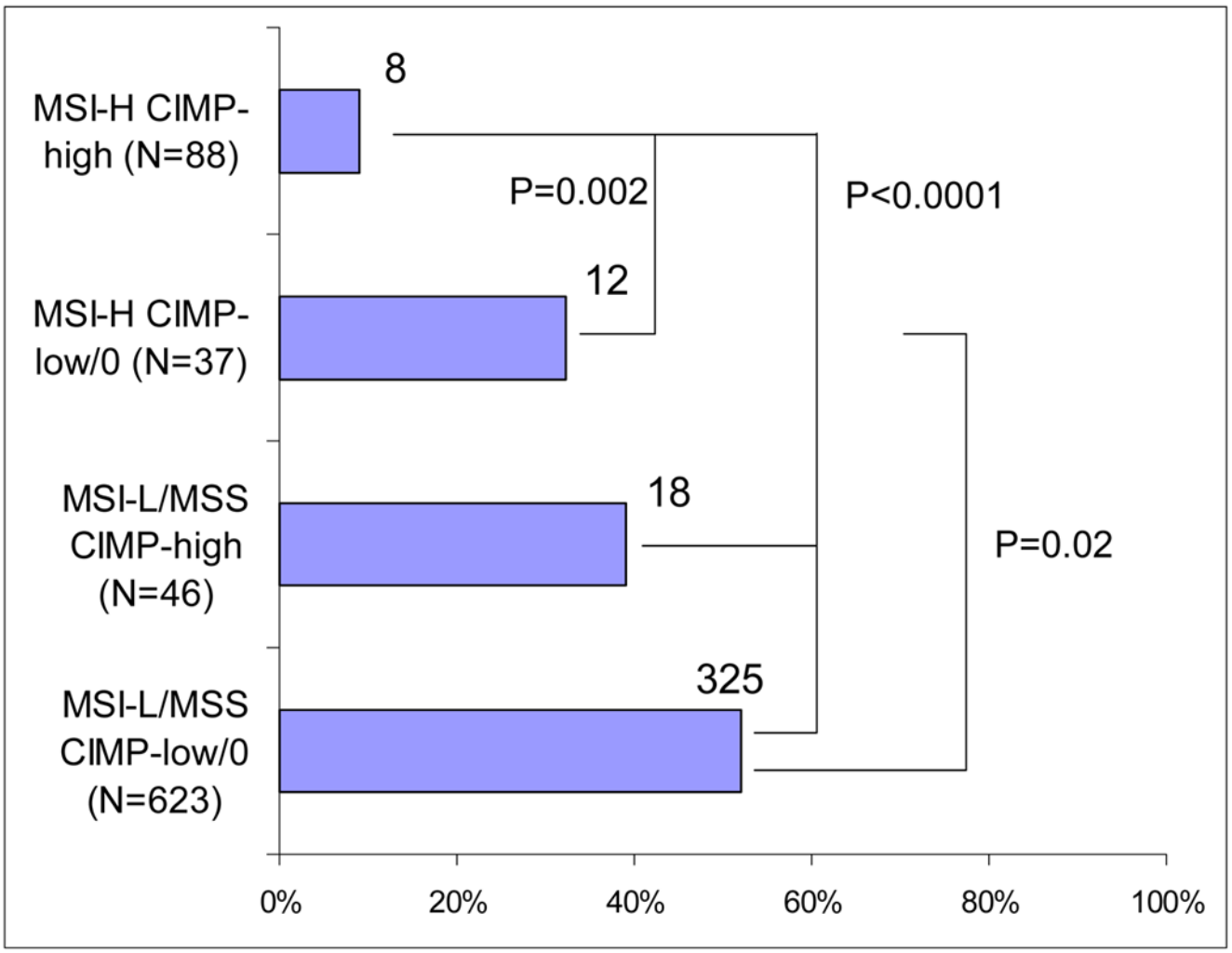

Figure 4. Frequency of cytoplasmic p27+ in various MSI/CIMP subtypes of colorectal cancer

MSI-H and CIMP-high are synergistically inversely associated with cytoplasmic p27 expression. CIMP, CpG island methylator phenotype; MSI, microsatellite instability; MSS, microsatellite stable.

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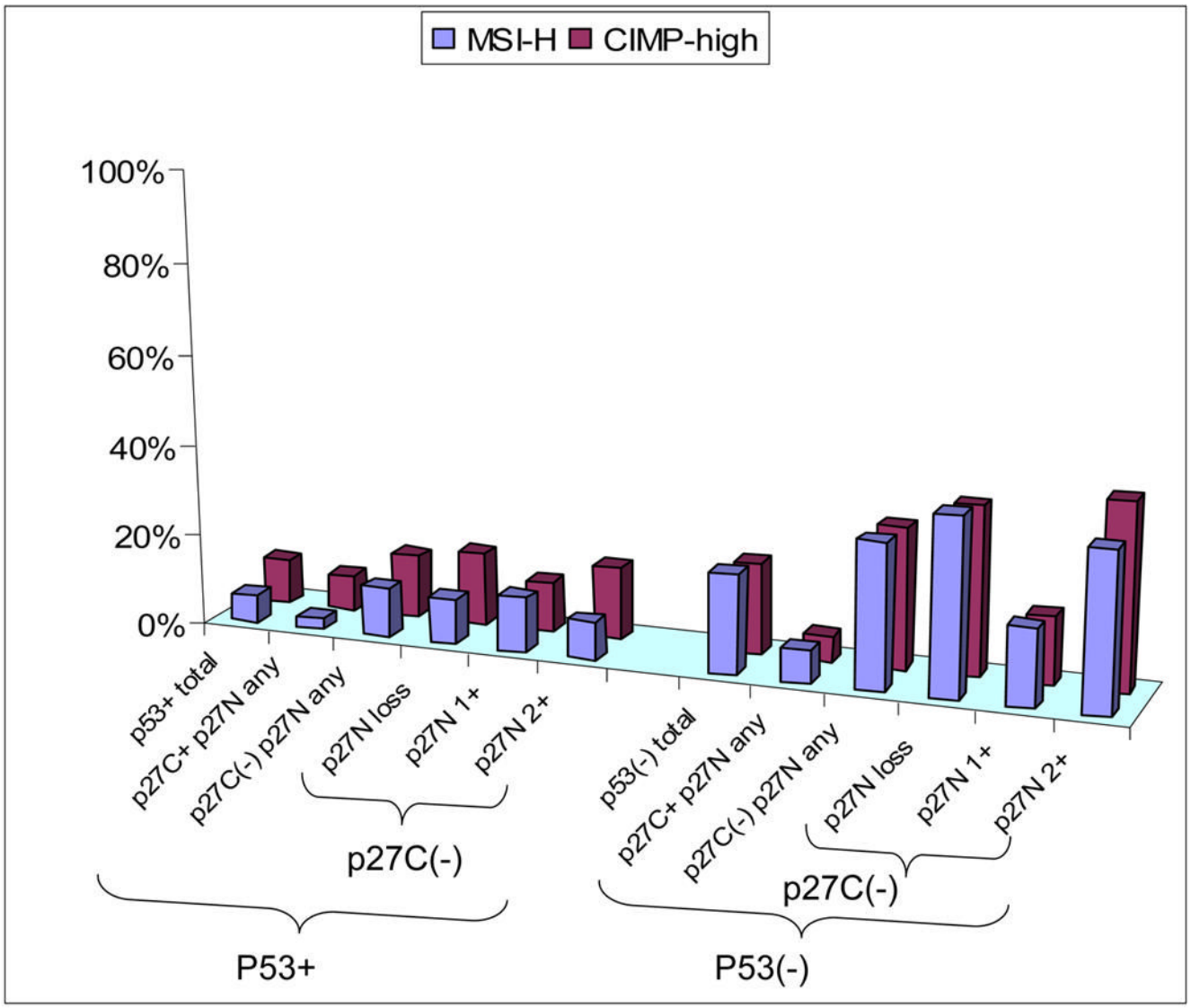

Figure 5. Frequency of MSI-H and CIMP-high in colorectal cancer with various combined p53/p27 status

The inverse association between cytoplasmic p27 and MSI-H (or CIMP-high) is much more pronounced in p53 negative tumors than in p53 positive tumors. For raw data, see Supplemental material 2. CIMP, CpG island methylator phenotype; MSI-H, microsatellite instability-high; p27C, p27 cytoplasmic; p27N, p27 nuclear.

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Table 1

Frequencies of MSI-H and CIMP-high in Tumors with Various Cytoplasmic and Nuclear p27 Expressions

p27 Cytoplasmic	p27 Nuclear	MSI-H		CIMP-high	
		Total 15% (127/853)		Total 16% (132/833)	
Positive	Any	5.1% (21/408)	P<0.0001	6.6% (26/392)	P<0.0001
Negative	Any	24% (106/445)		24% (106/441)	
Negative	Loss	29% (69/234)	P=0.001	30% (69/231)	P<0.0001
	1+	15% (23/154)		13% (19/151)	
	2+	25% (14/57)		31% (18/59)	P=0.002

Abbreviations: CIMP, CpG island methylator phenotype; MSI, microsatellite instability.

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Table 2

Frequencies of *KRAS* and *BRAF* Mutations in Tumors with Various Cytoplasmic and Nuclear p27 Expressions

p27 Cytoplasmic	p27 Nuclear	*KRAS* mutations	*BRAF* mutations	
		Total 37% (293/800)	Total 13% (108/800)	
Positive	Any	38% (146/381)	6.6% (25/381)	P<0.0001
Negative	Any	35% (147/419)	20% (83/419)	
Negative	Loss	33% (73/219)	24% (52/219)	P=0.04
	1+	36% (53/146)	15% (22/146)	
	2+	39% (21/54)	17% (9/54)	