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Clinical significance and prognostic value of Chromatin Assembly Factor-1 overexpression in human solid tumors

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Running title: CAF-1 prognostic marker in solid tumors

Keywords: chromatin assembly, carcinoma, marker, prognosis, proliferation.

Abstract

Aims: The chromatin assembly factor CAF-1, whose function is critical for maintaining chromatin stability during DNA replication and repair, has been identified as a proliferation marker in breast cancer. Here, we investigate the interest of CAF-1 as a proliferation marker in a wide spectrum of solid tumors, and assess its potential value in predicting clinical outcome.

Methods and Results: Using immunocytochemistry on paraffin-embedded tissue sections, we compare CAF-1 labeling index with known proliferation markers Ki-67 and MCMs, and we analyze its association with clinicopathological data and patients' outcome. CAF-1 expression shows a strong positive correlation with Ki-67, routinely used to detect proliferating cells, while it generally displays weaker correlations with MCM markers, known to label cells with replicative potential. CAF-1 expression is significantly associated with histological grade in breast, cervical, endometrial and renal cell carcinomas, and with disease stage in endometrial and renal carcinomas. Furthermore, high expression of CAF-1 is an independent predictor of adverse clinical outcome in renal, endometrial and cervical carcinomas.

Conclusions: These data demonstrate that CAF-1 is a proliferation marker in various malignant tumors with prognostic value in renal, endometrial and cervical carcinomas, which supports the relevance of CAF-1 as a clinical marker to monitor cancer progression.

Introduction

Cancer is a major cause of death in developed countries ¹. Early tumor detection as well as appropriate therapeutic decisions based on predicted clinical outcome and response to treatment are of key importance to treat cancer patients. Prediction of clinical outcome is generally based on the anatomical extent of disease (stage) and the degree of tumor differentiation (grade). In addition, molecular biomarkers ² including proliferation markers ³ can be used for cancer diagnosis and prognosis. A common feature of cancer pathogenesis is indeed uncontrolled and rapid cell proliferation ⁴. Accordingly, genes regulating cell proliferation have been observed to be overexpressed in the vast majority of tumor cells ³.

Not only genetic alterations but also abnormalities at the chromatin level can contribute to the aberrant pattern of gene expression that promotes proliferation of cancer cells ⁵⁻⁷. Chromatin abnormalities have even been proposed to be an early and seminal event for cancer progression ⁸, and anti-cancer treatments targeting chromatin components are in development ⁹. While active research is aimed at a better understanding of distinct chromatin defects during tumoral progression, Chromatin alterations are certainly considered as valuable markers of malignant transformation ^{10 11}, which may also provide prognostic information (reviewed in ¹²).

The structural organization of DNA with histone proteins into chromatin ¹³ is indeed of key importance for the regulation of gene expression, and maintenance of such an organization is thus crucial to preserve genome integrity and cellular identity ¹⁴. Chromatin assembly ¹⁵ involves specific factors called histone chaperones that escort histone proteins up to their deposition onto DNA ^{16, 17}. Both during replication ¹⁸ and repair events ¹⁹, chromatin assembly is coupled to DNA synthesis. The only chaperone known so far that is able to promote this activity is called CAF-1 (Chromatin Assembly Factor-1), a trimeric nuclear

complex²⁰, made of p150, p60²¹ and p48 subunits²² in human cells. Recent studies have highlighted CAF-1 intimate relationship with cell proliferation. In particular, CAF-1 expression is massively downregulated in quiescent cells²³ and appears essential for human cell proliferation²⁴⁻²⁶. Furthermore, we previously validated the use of CAF-1 p60 expression as a proliferation marker of clinical value in breast cancer²³. Here, we sought to extend these findings to a series of different human solid tumors and test the validity of CAF-1 p60 expression as a prognostic factor.

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Materials and methods

Selection of cases

Archival formalin-fixed, paraffin-embedded tissues and clinical material of common solid tumors including renal, colon, gastric, thyroid, pancreas, prostate, cervical, endometrial and breast cancer cases were provided by the First Department of Pathology of the University of Athens, which is a referral facility for the four University Hospitals in Athens, Greece. Selected patients correspond to consecutive cases of primary invasive tumors selected from an electronic medical record and diagnosed between 1994 and 2005, depending on the tumor type. All patients were examined routinely, according to institutional guidelines for each cancer type. The data reviewed, recorded in the hospital files, included baseline clinical and pathological data (available in Supporting information, Supplementary Table S1), the occurrence and the cause of death. Data were extracted in 2006. Immunohistochemical studies were performed on tissues obtained at surgery for cancer diagnosis. None of the patients had received either chemotherapy or radiotherapy before surgery. Patients received appropriate treatment afterwards, following standard procedures based on disease stage. Each patient had given informed consent and the study was approved by the ethical committee of the Medical School of University of Athens.

Immunohistochemical staining

Immunostainings for p60, Ki-67 and MCMs were performed on formalin-fixed paraffin embedded tissue sections (4 μ m) as previously described²³ using the corresponding antibodies: CAF-1 p60 (mAb8133, Abcam), Ki-67 (MIB1, DAKO) and MCM proteins (MCM-2: MCA1859; MCM-5: MCA1860, Serotec).

Statistical analyses

The percentages of positively stained cells in immunocytochemistry experiments (labeling indexes) were obtained by scoring at least 1000 cells in each case by two independent observers, and the mean values were used for the following statistical analyses (S-Plus 2000 software, MathSoft Inc.). Correlations between continuous variables were studied using Spearman's rank correlation test. For multiple group comparisons of continuous variables the non parametric Kruskal-Wallis test was employed. Comparisons of labeling indexes obtained with two different markers between paired samples were performed by the non parametric Wilcoxon's signed-rank test. Statistical significance was taken as $p < 0.05$. Survival was assessed through medical records and defined as cause-specific survival, calculated from the date of surgical excision of the primary tumor to the date of death due to cancer or last follow-up. Survival data were systematically collected on the date of data extraction by telephone call to the patient. One case was censored because mortality was due an accident. Losses-to-follow-up were limited to an acceptable percentage of cases except in cervical cancer, where we verified that it did not bias the analysis since baseline characteristics of patients with or without survival data were not statistically different (Table S1). Survival curves were derived from Kaplan-Meier estimates. The association of CAF-1 p60 expression with clinical outcome was determined by univariate and multivariate Cox regression models with forward stepwise inclusion of factors and an inclusion criterion of $p < 0.20$. CAF-1 p60 expression was introduced as continuous variable and as categorical variable in the models. Categorical variable was obtained by performing a median split of distribution. Due to the strong correlation between CAF-1 p60 and Ki-67, the latter was excluded from multivariate analyses.

Results

CAF-1 is a proliferation marker in various human carcinomas

In order to test the validity of CAF-1 expression as a proliferation marker in solid tumors, we performed immunohistochemical detection of CAF-1 p60 on a series of paraffin-embedded tissue sections including breast (n=79), cervical (n=64), endometrial (n=62), renal (n=55), prostate (n=43), gastric (n=64), colon (n=108), pancreatic (n=47) and thyroid (n=52) carcinoma cases (Figure 1A). In each cancer type, we tested the correlation of CAF-1 p60 expression with the expression of the known marker of proliferation Ki-67²⁷. We also analyzed the association of CAF-1 expression with clinicopathological variables important for patients' care and their final outcome (listed in Supporting information, Supplementary Table S1), in particular stage and grade.

Strong positive correlations were observed between CAF-1 p60 and Ki-67 labeling indexes in all tumors examined (from $r=0.80$ in cervical cancer to $r=0.99$ in pancreatic cancer, Figure 1B), demonstrating that CAF-1 is a proliferation marker of general value in human carcinomas. A consistently weaker correlation, although still significant, was observed with MCM proliferation markers²⁸⁻³⁰ as exemplified in gastric cancer (Figure 2A). Interestingly, MCM markers also commonly display significantly higher labeling indexes than CAF-1 p60 ($p<0.001$ in colon, gastric and thyroid cancer, Figure 2B) while the fraction of cells detected with CAF-1 p60 and Ki-67 is comparable (based on Wilcoxon's signed-ranked test, data not shown).

Furthermore, CAF-1 p60 expression was significantly associated with histological grade in breast, cervical, endometrial and renal cell carcinoma ($p=0.016$, $p=0.037$, $p=0.0006$, $p=0.0006$, respectively), and with disease stage in endometrial and renal cell carcinoma

($p=0.012$ and $p=0.015$, respectively). No significant associations with any other clinicopathological data examined were noted in our study.

Taken together, these data demonstrate that CAF-1 is a proliferation marker of clinical significance in a series of human solid tumors.

CAF-1 expression is associated with adverse clinical outcome in a subset of tumor types

The significant association of CAF-1 p60 expression with tumor stage and grade at least in a subset of tumor types prompted us to analyse the potential prognostic value of CAF-1 marker. We therefore examined the association between CAF-1 p60 expression and cause-specific patient survival by Kaplan-Meier analysis and Cox regression models on a series of patients with median follow-up ranging from 8 months for pancreatic cancer to 81 months for cervical cancer (Supporting information, Supplementary Table S1). Interestingly, CAF-1 p60 expression stood out as an independent predictor of survival in renal, endometrial and cervical cancers (Table 1, Figure 3). High levels of CAF-1 p60 immunostaining were indeed associated with poor clinical outcome in these cancer types (hazard ratio for death, 4.4, 5.5 and 3.3, with $p=0.006$, 0.03 and 0.04 for renal, endometrial and cervical tumors, respectively), based on multivariate Cox regression analyses (Table 1, Figure 3). The most significant differences were observed in renal cancer with 16 deaths in 22 patients with high p60 staining (median survival 8 months, range 2-66 months) compared with 7 deaths in 29 patients with low p60 staining (median survival 17 months, range 3-144 months). Our analyses did not show any significant prognostic value of CAF-1 detection in colon ($p=0.64$), gastric ($p=0.43$) and pancreatic ($p=0.23$) cancer. Univariate Cox regression analyses showed similar trends with CAF-1 p60 expression introduced as continuous variable in the models

(data not shown). Longer follow-up of patients will be required to examine CAF-1 prognostic value in slowly progressing breast, prostate and thyroid cancers.

Taken together, our data highlight the tight association of CAF-1 p60 expression with the proliferative state of the cells in various human solid tumors, validating its use as a general proliferation marker. Additionally, CAF-1 p60 level proved of prognostic significance in a subset of tumor types, namely renal, cervical and endometrial. These new data extend the clinical value of CAF-1 to assess cancer progression.

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Discussion

In this study, we have shown that the expression of the chromatin assembly factor CAF-1 is a clinically relevant indicator of cell proliferation in human carcinomas, thus confirming and extending our previous findings in breast cancer cases²³. This property most likely relates to CAF-1 function coupled to DNA replication. Given the additional role of CAF-1 in chromatin assembly coupled to DNA repair^{19, 31-35}, it would also be worthwhile investigating its potential utility in monitoring/predicting tumor response to therapy. This requires a detailed analysis of CAF-1 expression levels in cells exposed to different sources of DNA damage, an issue that has begun to be considered only in one study³⁶.

Our analyses also reveal that CAF-1 displays significant but notably weak correlations with MCM proliferation markers, which generally label a larger proportion of cells, suggesting that they detect distinct cell populations. This is consistent with the idea that MCMs mark cells with replicative potential in addition to actively proliferating cells³⁷ while CAF-1 only labels the latter. A combination of both MCM and CAF-1 markers would thus be most useful to consider in difficult cases since MCM markers seem more sensitive and CAF-1 more discriminative.

In addition to its clinical utility for refining cancer diagnosis, CAF-1 marker also displays prognostic value, at least in a subset of the tumor types analysed (in particular in renal carcinomas), which can be useful for guiding therapeutic decisions. Interestingly, CAF-1 p60 was also recently put forward as a marker of prognostic value in prostate cancer and squamous cell carcinoma of the tongue^{38, 39}, which further expands the list of human carcinomas in which high CAF-1 p60 expression is associated with adverse clinical outcome.

Elucidation of the biological role of CAF-1 in cell proliferation and tumorigenesis is also of major interest. In particular, whether CAF-1 overexpression has oncogenic potential, as recently described for replication licensing factors⁴⁰, remains to be examined. Furthermore, assessment of CAF-1 expression in early pre-cancerous lesions would also help clarifying when CAF-1 overexpression occurs in the course of cancer development.

Our results also need to be considered in light of the emerging concept of cancer stem cells⁴¹, which refers to a small subpopulation of tumor cells that is required for tumor growth and therefore constitutes the main target of anti-cancer therapies. It would be interesting to compare CAF-1 expression in cancer stem cells relative to other cells within the tumor to determine if this marker is also associated with the cancer stem cell phenotype and if it could contribute to more sensitive detection methods for cancer stem cells. Another population of cells in which assesment of CAF-1 expression would be informative is stromal fibroblasts of the tumor microenvironment, which are known to potentiate tumor development⁴². These cells have indeed been reported to differ from their normal counterparts in terms of proliferation/migration capacities, which relates to differences in gene expression arising from chromatin changes rather than genetic alterations⁴³⁻⁴⁵.

Finally, in light of recent genome-wide analyses of chromatin marks that identify specific alterations in human cancer cells (reviewed in⁴⁶), our study underlines that a better understanding of chromatin-related events associated with tumorigenesis can prove useful for cancer diagnosis and prognosis.

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References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, et al. Cancer statistics, 2008. *CA Cancer J Clin* 2008;**58**(2):71-96.
2. Sawyers CL. The cancer biomarker problem. *Nature* 2008;**452**(7187):548-52.
3. Whitfield ML, George LK, Grant GD, Perou CM. Common markers of proliferation. *Nat Rev Cancer* 2006;**6**(2):99-106.
4. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;**100**(1):57-70.
5. Esteller M. Epigenetics provides a new generation of oncogenes and tumour-suppressor genes. *Br J Cancer* 2007;**96** Suppl:R26-30.
6. Iacobuzio-Donahue CA. Epigenetic Changes in Cancer. *Annu Rev Pathol* 2008.
7. Jones PA, Baylin SB. The epigenomics of cancer. *Cell* 2007;**128**(4):683-92.
8. Baylin SB, Ohm JE. Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction? *Nat Rev Cancer* 2006;**6**(2):107-16.
9. Yoo CB, Jones PA. Epigenetic therapy of cancer: past, present and future. *Nat Rev Drug Discov* 2006;**5**(1):37-50.
10. Fraga MF, Ballestar E, Villar-Garea A, Boix-Chornet M, Espada J, Schotta G, et al. Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. *Nat Genet* 2005;**37**(4):391-400.
11. Ozdag H, Teschendorff AE, Ahmed AA, Hyland SJ, Blenkiron C, Bobrow L, et al. Differential expression of selected histone modifier genes in human solid cancers. *BMC Genomics* 2006;**7**:90.
12. Kurdistani SK. Histone modifications as markers of cancer prognosis: a cellular view. *Br J Cancer* 2007;**97**(1):1-5.
13. Kornberg RD. Structure of chromatin. *Annu Rev Biochem* 1977;**46**:931-54.

14. Groth A, Rocha W, Verreault A, Almouzni G. Chromatin challenges during DNA replication and repair. *Cell* 2007;**128**(4):721-33.
15. Polo SE, Almouzni G. Chromatin assembly: a basic recipe with various flavours. *Curr Opin Genet Dev* 2006;**16**(2):104-11.
16. Corpet A, Almouzni G. Making copies of chromatin: the challenge of nucleosomal organization and epigenetic information. *Trends Cell Biol* 2009;**19**(1):29-41.
17. De Koning L, Corpet A, Haber JE, Almouzni G. Histone chaperones: an escort network regulating histone traffic. *Nat Struct Mol Biol* 2007;**14**(11):997-1007.
18. Stillman B. Chromatin assembly during SV40 DNA replication in vitro. *Cell* 1986;**45**(4):555-65.
19. Gaillard PH, Martini EM, Kaufman PD, Stillman B, Moustacchi E, Almouzni G. Chromatin assembly coupled to DNA repair: a new role for chromatin assembly factor I. *Cell* 1996;**86**(6):887-96.
20. Smith S, Stillman B. Purification and characterization of CAF-I, a human cell factor required for chromatin assembly during DNA replication in vitro. *Cell* 1989;**58**(July 14):15-25.
21. Kaufman PD, Kobayashi R, Kessler N, Stillman B. The p150 and p60 subunits of chromatin assembly factor I - a molecular link between newly synthesized histones and DNA replication. *Cell* 1995;**81**(7):1105-1114.
22. Verreault A, Kaufman PD, Kobayashi R, Stillman B. Nucleosome assembly by a complex of CAF-1 and acetylated histones H3/H4. *Cell* 1996;**87**(1):95-104.
23. Polo SE, Theocharis SE, Klijanienko J, Savignoni A, Asselain B, Vielh P, et al. Chromatin assembly factor-1, a marker of clinical value to distinguish quiescent from proliferating cells. *Cancer Res* 2004;**64**(7):2371-81.
24. Nabatiyan A, Krude T. Silencing of chromatin assembly factor 1 in human cells leads to cell death and loss of chromatin assembly during DNA synthesis. *Mol Cell Biol* 2004;**24**(7):2853-62.

25. Ye X, Franco AA, Santos H, Nelson DM, Kaufman PD, Adams PD. Defective S phase chromatin assembly causes DNA damage, activation of the S phase checkpoint, and S phase arrest. *Mol Cell* 2003;**11**:341-351.
26. Hoek M, Stillman B. Chromatin assembly factor 1 is essential and couples chromatin assembly to DNA replication in vivo. *Proc Natl Acad Sci U S A* 2003;**100**:12183-12188.
27. Brown DC, Gatter KC. Monoclonal antibody Ki67: its use in histopathology. *Histopathology* 1990;**17**:489-503.
28. Tachibana KE, Gonzalez MA, Coleman N. Cell-cycle-dependent regulation of DNA replication and its relevance to cancer pathology. *J Pathol* 2005;**205**(2):123-9.
29. Freeman A, Morris LS, Mills AD, Stoeber K, Laskey RA, Williams GH, et al. Minichromosome Maintenance Proteins as Biological Markers of Dysplasia and Malignancy. *Clin. Cancer Res.* 1999;**5**:2121-2132.
30. Gonzalez MA, Tachibana KE, Laskey RA, Coleman N. Control of DNA replication and its potential clinical exploitation. *Nat Rev Cancer* 2005;**5**(2):135-41.
31. Green CM, Almouzni G. Local action of the chromatin assembly factor CAF-1 at sites of nucleotide excision repair in vivo. *Embo J* 2003;**22**(19):5163-74.
32. Martini E, Roche DM, Marheineke K, Verreault A, Almouzni G. Recruitment of phosphorylated chromatin assembly factor 1 to chromatin after UV irradiation of human cells. *J Cell Biol* 1998;**143**(3):563-75.
33. Polo SE, Roche D, Almouzni G. New Histone Incorporation Marks Sites of UV Repair in Human Cells. *Cell* 2006;**127**(3):481-93.
34. Lan L, Nakajima S, Oohata Y, Takao M, Okano S, Masutani M, et al. In situ analysis of repair processes for oxidative DNA damage in mammalian cells. *Proc Natl Acad Sci U S A* 2004;**101**(38):13738-43.
35. Okano S, Lan L, Caldecott KW, Mori T, Yasui A. Spatial and temporal cellular responses to single-strand breaks in human cells. *Mol Cell Biol* 2003;**23**(11):3974-81.

36. Nabatiyan A, Szuts D, Krude T. Induction of CAF-1 expression in response to DNA strand breaks in quiescent human cells. *Mol Cell Biol* 2006;**26**(5):1839-49.
37. Stoeber K, Tlsty TD, Happerfield L, Thomas GA, Romanov S, Bobrow L, et al. DNA replication licensing and human cell proliferation. *J Cell Sci* 2001;**114**(Pt 11):2027-41.
38. Staibano S, Mascolo M, Mancini FP, Kisslinger A, Salvatore G, Di Benedetto M, et al. Overexpression of chromatin assembly factor-1 (CAF-1) p60 is predictive of adverse behaviour of prostatic cancer. *Histopathology* 2009.
39. Staibano S, Mignogna C, Lo Muzio L, Mascolo M, Salvatore G, Di Benedetto M, et al. Chromatin assembly factor-1 (CAF-1)-mediated regulation of cell proliferation and DNA repair: a link with the biological behaviour of squamous cell carcinoma of the tongue? *Histopathology* 2007;**50**(7):911-9.
40. Lontos M, Koutsami M, Sideridou M, Evangelou K, Kletsas D, Levy B, et al. Deregulated overexpression of hCdt1 and hCdc6 promotes malignant behavior. *Cancer Res* 2007;**67**(22):10899-909.
41. Ward RJ, Dirks PB. Cancer stem cells: at the headwaters of tumor development. *Annu Rev Pathol* 2007;**2**:175-89.
42. Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. *Nature* 2004;**432**(7015):332-7.
43. Qiu W, Hu M, Sridhar A, Opeskin K, Fox S, Shipitsin M, et al. No evidence of clonal somatic genetic alterations in cancer-associated fibroblasts from human breast and ovarian carcinomas. *Nat Genet* 2008;**40**(5):650-5.
44. Hu M, Yao J, Cai L, Bachman KE, van den Brule F, Velculescu V, et al. Distinct epigenetic changes in the stromal cells of breast cancers. *Nat Genet* 2005;**37**(8):899-905.
45. Allinen M, Beroukhim R, Cai L, Brennan C, Lahti-Domenici J, Huang H, et al. Molecular characterization of the tumor microenvironment in breast cancer. *Cancer Cell* 2004;**6**(1):17-32.

46. Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat Rev Genet* 2007;**8**(4):286-98.

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Figure legends

Figure 1 : Comparison between CAF-1 p60 and Ki-67 labeling indexes in various solid tumors.

A. Immunohistochemical staining for CAF-1 p60 and Ki-67 in breast and colon cancer specimens (original magnification X 200).

B. Correlations between CAF-1 p60 and Ki-67 labeling indexes in various human carcinomas. r =Spearman's rank correlation coefficient; linear regression line is represented; N = number of cases; All p values are $<10^{-4}$.

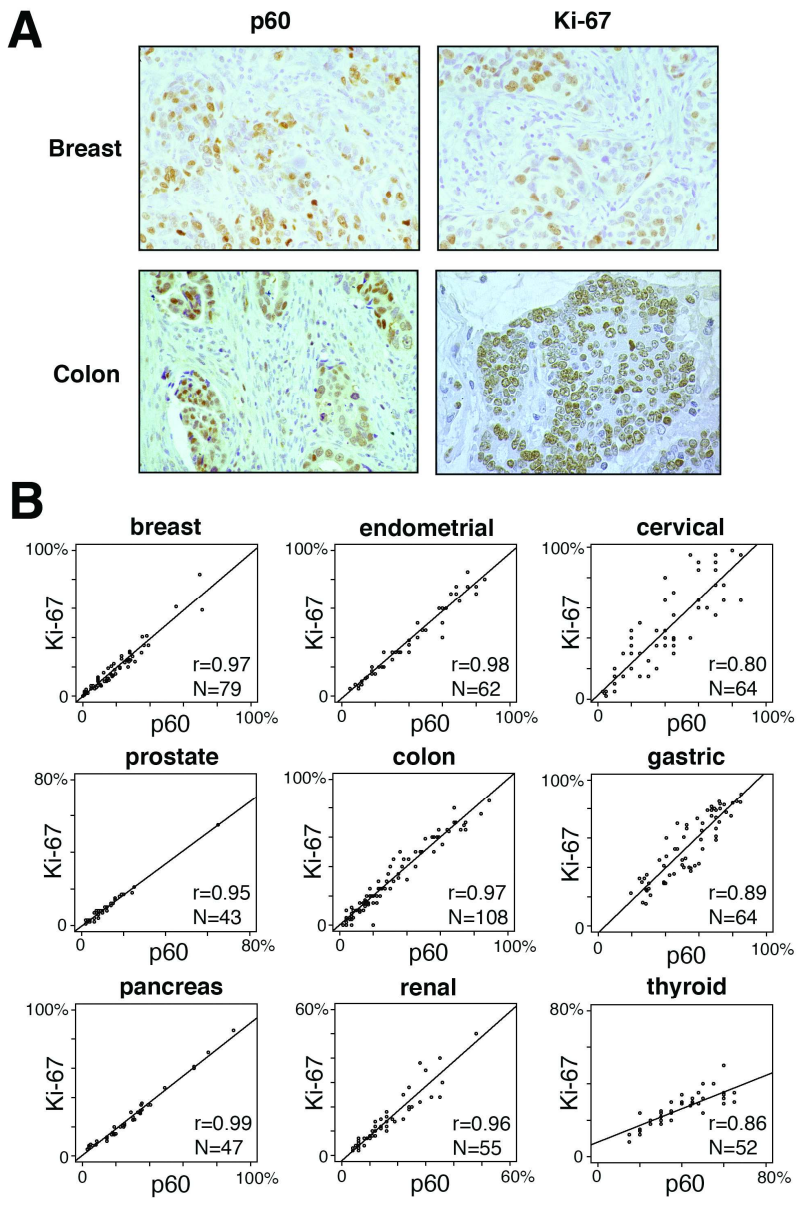
Figure 2: Comparison between CAF-1 p60 and MCMs labeling indexes.

A. Correlations between CAF-1 p60 and MCMs labeling indexes in gastric cancer. r = Spearman's rank correlation coefficient; linear regression line is represented; N = Number of cases; All p values are $<10^{-4}$.

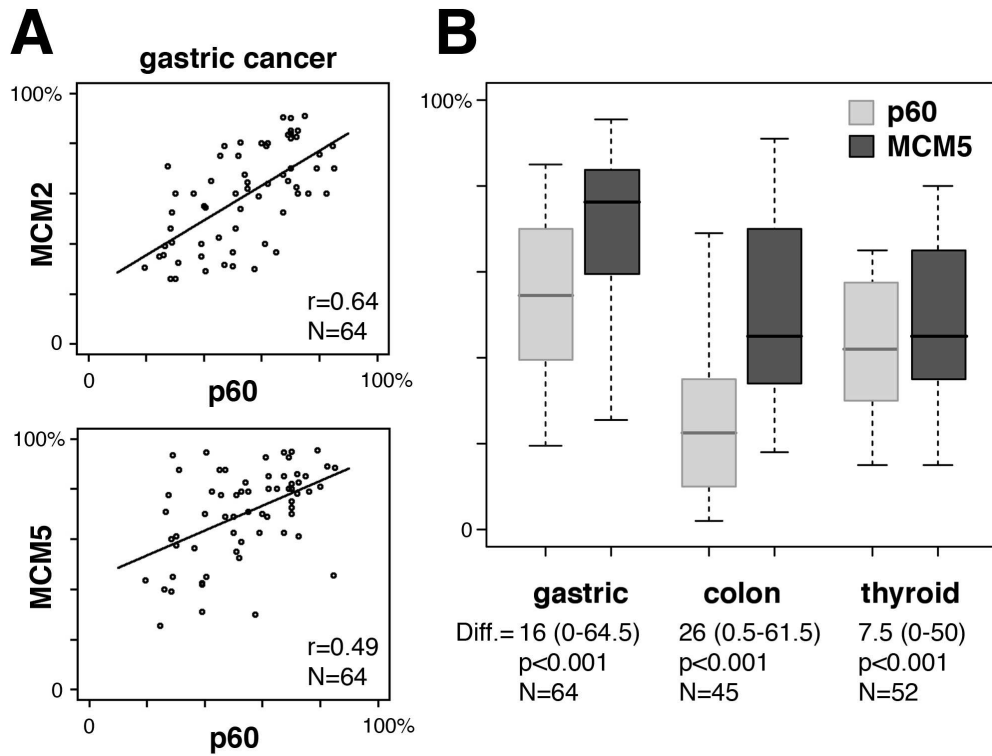
B. Boxplot representation of p60 and MCM5 labeling indexes in the indicated tumors. Box: 25th-75th percentile; horizontal line inside the box: median; dotted line: range; Diff.: difference between MCM5 and p60 labeling indexes presented as median (minimum-maximum); N : number of cases. Significance as determined by the Wilcoxon's signed-rank test is shown.

Figure 3 : Association of CAF-1 p60 expression level with clinical outcome.

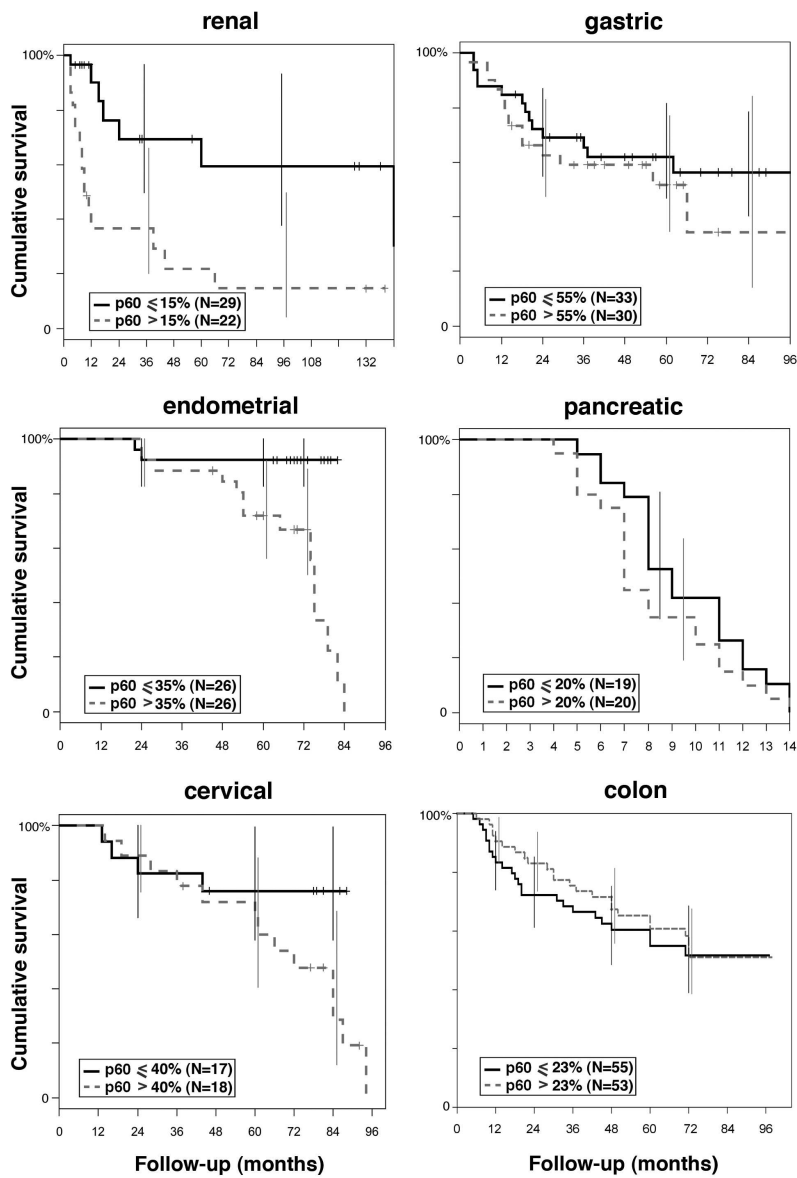
Kaplan-Meier survival curves represent patients' cause-specific survival according to p60 levels. Patients are stratified in two groups based on the median of p60 labeling index as a cut-off (dashed line: high p60 labeling index; solid line: low p60 labeling index). Error bars represent 95% confidence intervals. N : number of cases.



170x249mm (300 x 300 DPI)



169x132mm (300 x 300 DPI)



168x247mm (300 x 300 DPI)

Table 1 : CAF-1 p60 prognostic value (prediction of cause-specific survival) determined by univariate and multivariate Cox regression models in the indicated tumors.

Cancer	N	UNIVARIATE			MULTIVARIATE		
		HR	95% C.I.	p	HR	95% C.I.	p
Renal	51						
p60=<15%	29	1			1		
p60>15%	22	4.9	[1.9 - 12.6]	<0.001	4.4	[1.4 - 14.0]	0.006*
Endometrial	52						
p60=<35%	26	1			1		
p60>35%	26	8.7	[1.9 - 38.7]	0.005	5.2	[1.1 - 25.6]	0.03†
Cervical	35						
p60=<40%	17	1			1		
p60>40%	18	2.6	[0.8 - 8.1]	0.08§	3.3	[1.0 - 10.6]	0.04‡
Gastric	63				-	-	NS
p60=<55%	33	1					
p60>55%	30	1.4	[0.6 - 2.9]	0.43			
Pancreatic	39				-	-	NS
p60=<20%	19	1					
p60>20%	20	1.5	[0.8 - 2.8]	0.23			
Colon	108				-	-	NS
p60=<23%	55	1					
p60>23%	53	0.9	[0.5 - 1.5]	0.64			

In addition of p60, forward stepwise inclusions were performed with:

* age (not included in the final model), TNM (p=0.003) and Fuhrman grade (p=0.003)

† histological grade (not included in the final model) and FIGO stage (p=0.005)

‡ histological grade and lymph node invasion (global p-value=0.009)

§ In the cervical cancer cases analysed in our study, squamous histological type that has good prognosis is more frequently, although not significantly, associated with high p60 values than adenocarcinoma type, an indicator of bad prognosis. This explains why p60 shows a

significant association with clinical outcome in multivariate analysis only, while its effect is masked by histological type in univariate analysis.

Renal cancer variables	N	UNIVARIATE			MULTIVARIATE		
		HR	95% C.I.	p	HR	95% C.I.	p
sex				0.96			
male	34	1					
female	17	1.0	[0.4 - 2.5]				
age (years)				0.03			
>60	25	1					
<=60	26	0.4	[0.1 - 0.9]				
TNM				0.001			0.003
T1N0, T2N0	22	1			1		
T2N+	2	12.1	[1.9 - 75.8]		28.9	[3.8 - 221]	
T3N0	18	2.8	[0.8 - 10.5]		0.8	[0.2 - 3.8]	
T3N+	3	12.6	[2.4 - 65.7]		2.8	[0.4 - 17.2]	
T4N0, T4N+	6	12.5	[3.0 - 51.7]		5.0	[1.1 - 22.8]	
grade				<0.001			0.003
I, II	24	1			1		
III, IV	26	4.8	[1.8 - 13.2]		5.8	[1.6 - 20.5]	
p60 L.I.				<0.001			0.006
<=15	29	1			1		
>15	22	4.9	[1.9 - 12.6]		4.4	[1.4 - 14.0]	

Patients are stratified in two groups based on the median of p60 labeling index as a cut-off (also see Figure 3). N : number of cases ; HR : Hazard Ratio ; C.I. : Confidence Interval ; L.I. : Labeling Index ; NS : Not Significant.

Table S1 : Clinico-pathological data.

Values are presented as median [minimum-maximum] for continuous variables and number of patients (N, %) for categorical variables.

BREAST			ENDOMETRIAL			CERVICAL			GASTRIC		
N=80			N=62			N=64			N=64		
Age (years)	56	[22-88]	Follow-up (months)	70	[45-84]	Follow-up (months)	81	[36-94]	Follow-up (months)	57	[15-126]
			Age (years)	65	[40-82]	Age (years)* (3)	53.5	[30-91]	Age (years)	67	[39-88]
	N	%		N	%		N	%		N	%
Histological type	80		Histological type	62		Histological type* (1)	64		Sex	64	
ductal	55	69	adenocarcinoma	49	79	adenocarcinoma	10	16	Female	17	27
lobular	0	0	squamous component	13	21	squamous	46	72	Male	47	73
mixed	3	4	Histological grade	60		mixed type	8	12	T	63	
fibroadenoma	6	7	well differentiated	16	27	Histological grade* (1)	62		T1	9	14
fibrocystic disease	16	20	moderately	37	62	well differentiated	9	15	T2	23	37
Histological grade	57		poorly	7	11	moderately	43	69	T3	28	44
I	7	12	FIGO stage	62		poorly	10	16	T4	3	5
II	34	60	1a	5	8	FIGO stage* (1)	63		N	64	
III	16	28	1b	21	34	1a	6	10	N0	27	42
T	57		1c	26	42	1b	55	87	N1	34	53
T1	23	40	2a	3	5	2a	2	3	N2	3	5
T2	26	46	2b	2	3	Lymph node invas.* (1)	64		Stage	63	
T3, T4	8	14	3a	2	3	Yes	9	14	1a	6	9
N	58		4a	3	5	No	55	86	1b	12	19
N0	23	40	Status	52		Lymphatics invas.* (2)	63		2	25	40
N1	25	43	Dead	16	31	Yes	41	65	3a	14	22
N2	9	15	Alive	36	69	No	22	35	3b	3	5
N3	1	2				Vessel invasion* (2)	63		4	3	5
M	58					Yes	23	37	Histological type	63	
M0	56	97				No	40	63	Intestinal	33	52
M1	2	3				Status	35		Diffuse	27	43
						Dead	17	49	Mixed	3	5
						Alive	18	51	Histological grade	61	
									well differentiated	4	7
									moderately	32	52
									poorly	25	41
									Status	63	
									Dead	27	43
									Alive	36	57

* not statistically different between patients with or without follow-up data based on $p > 0.05$ with:

(1) Fisher's exact test

(2) Pearson's Chi-Squared test

(3) Wilcoxon rank sum test

PANCREAS			COLON			THYROID			RENAL		
N=47			N=108			N=52			N=55		
Follow-up (months)	8	[4-14]	Follow-up (months)	79	[6-98]	Follow-up (months)	48	[4-48]	Follow-up (months)	34	[5-144]
Age (years)	67	[46-84]	Age (years)	70	[34-94]	Age (years)	46	[14-78]	Age (years)	60.5	[33-80]
	N	%		N	%		N	%		N	%
T	47		Sex	108		Sex	52		Sex	55	
T1	4	8	Female	44	41	Female	38	73	Female	17	31
T2	6	13	Male	64	59	Male	14	27	Male	38	69
T3	30	64	Stage	108		T	50		TNM	55	
T4	7	15	A	20	19	T1	5	10	T1, T2N0	22	40
N	47		B	47	43	T2	23	46	T2N+	2	4
N0	24	51	C1	21	19	T3	11	22	T3N0	21	38
N1	23	49	C2	20	19	T4	11	22	T3N+	3	5
M	47		Grade	104		N	50		T4Nany	7	13
M0	47	100	I	10	10	N0	23	46	Histological type	51	
M1	0	0	II	79	76	N1	27	54	clear cell	38	74
Stage	47		III	15	14	M	50		papillary	9	18
IA	4	8.5	Vessel invasion	108		M0	47	94	chromophobe	3	6
IB	4	8.5	Yes	67	62	M1	3	6	sarcomatoid	1	2
IIA	13	28	No	41	38	Stage	52		Fuhrman grade	51	
IIB	19	40	Status	108		I	11	21	I	10	20
III	7	15	Dead	48	44	II	15	29	II	14	28
IV	0	0	Alive	60	56	III	21	40	III	17	34
Histological grade	46					IV	5	10	IV	8	18
I	7	15	PROSTATE	N=43		Histological type	52		Status	51	
II	31	67.5				Myeloid	35	67	Dead	23	45
III	8	17.5	Age (years)	69	[52-79]	Huertle cell	3	6	Alive	28	55
Status	39			N	%	Anaplastic	2	4			
Dead	39	100	Gleason's score	43		Papillary	12	23			
Alive	0	0	5	7	16	Status	52				
			6	6	14	Dead	6	12			
			7	21	49	Alive	46	88			
			8	5	12						
			9	4	9	T	43				
			T	43		1	3	7			
			1	3	7	2	16	37			
			2	16	37	3	23	54			
			3	23	54	4	1	2			
			4	1	2						