**Table 1.** Features of 13 cases of Fe-NTA-induced renal cell carcinomas.

| Tumor case | Size (mm) | Metastasis | Invasion | Nuclear atypia grade | Growth pattern | Copy number aberration at *Met* locus | Copy number aberration at *Cdkn2a/2b* locus |
|---|---|---|---|---|---|---|---|
| FB7-1 | 20 | None | None | Low | Intermediate | None | Loss |
| FB32-4 | 15 | Lung | None | Intermediate | Intermediate | None | Loss |
| FB7-7 | 60 | Lung | None | Intermediate | Expansive | Amplification | None |
| FB59-1 | 15 | Lung | Peritoneal | Intermediate | Infiltrating | None | HD |
| FB14-3 | 15 | None | None | High | Expansive | Amplification | Loss |
| FB28-7 | 30 | None | None | High | Intermediate | None | Loss |
| BF51-1 | 28 | None | None | High | Intermediate | Amplification | None |
| FB14-6 | 30 | Lung | Peritoneal | High | Intermediate | Amplification | Loss |
| FB21-2 | 40 | None | None | High | Infiltrating | Amplification | HD |
| FB45-4 | 40 | Lung | None | High | Infiltrating | Amplification | HD |
| FB30-5 | 60 | Lung | Peritoneal | High | Infiltrating | Amplification | Loss |
| FB33-7 | 70 | Lung | Peritoneal | High | Infiltrating | Amplification | None |
| BF57-5 | 25 | Lung | Peritoneal | High | Infiltrating | Amplification | HD |

Fe-NTA: ferric nitrilotriacetate; HD: homozygous deletion.
doi:10.1371/journal.pone.0043403.t001

or subtle genetic alterations (**Fig. S5**). Accordingly, oxidative stress, including that induced due to excess iron, could be one of the causes of human renal carcinogenesis. Indeed, numerous epidemiological studies have associated iron and steel industry workers with an increased RCC risk [35].

A frequency plot analysis revealed two remarkable features. First, the chromosomal aberrations showed a preference for loss against the ploidy of each cancer genome. Mostly, the aberration was represented by a deletion at either a whole chromosomal level (monosomy) or at a segmental level (**Fig. 1B**). The most common target for loss was the *Cdkn2a/2b* locus. The predominance of loss in the profile of chromosomal alterations may be attributed to the early stage of carcinogenesis. We previously demonstrated that cells with a hemizygous deletion at *Cdkn2a* appear as early as a few weeks after initiating a Fe-NTA administration [36].

Our present analysis revealed that the monoallelic loss of chromosome 5 in its entirety or of an equivalently wide region is the major first event. Indeed, we found only one case (6.7%) of a monoallelic loss of an extremely narrow region (~350 kilobases; **Figs. 2A and 2B**). Fe-NTA catalyzes the generation of hydroxyl radicals through a Fenton reaction specifically in the lumina of renal proximal tubules, which leads to degeneration and necrosis/apoptosis of those cells [37,38]. Because kidney is a vital organ that performs urea excretion, reabsorption of valuable molecules as

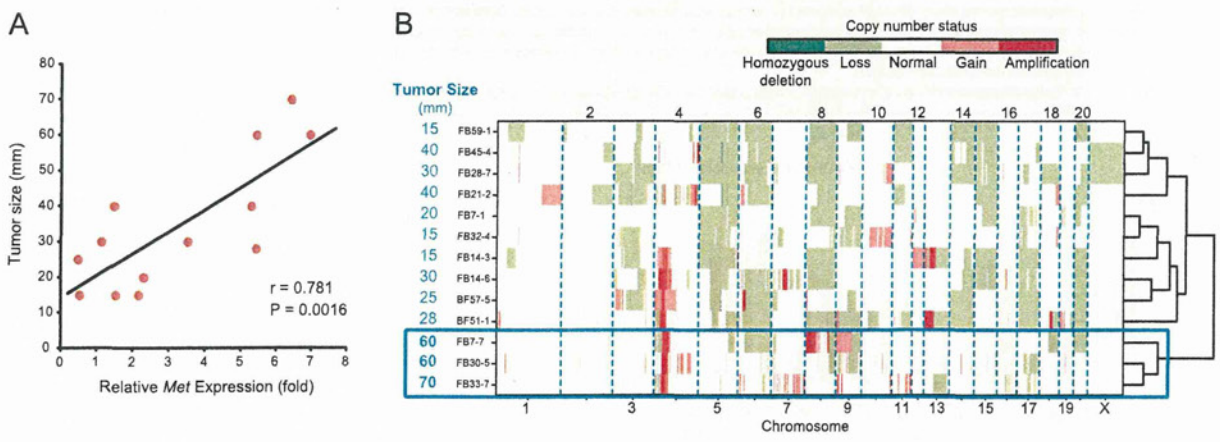


**Figure 4. Tumor sizes of Fe-NTA induced RCCs are controlled by the genetic features.** (A) *Met* expression is significantly correlated with tumor size. Pearson's correlation coefficient (r) and the corresponding P value are written on the plot area. (B) Hierarchical clustering of the RCC tumors based on the whole genome patterns of the copy number changes. The large-size tumors form a distinct cluster.
doi:10.1371/journal.pone.0043403.g004

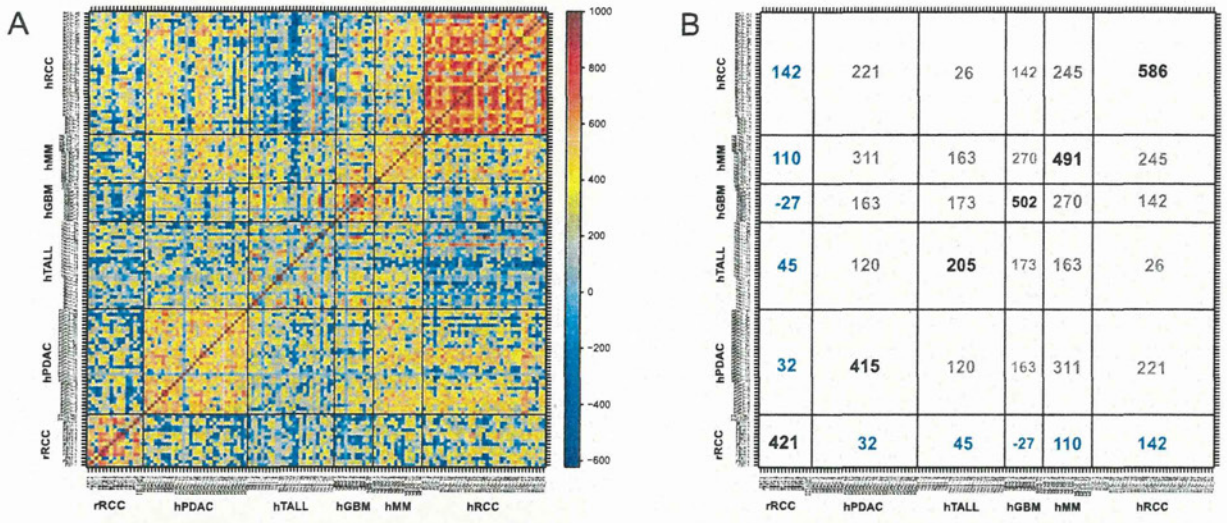


**Figure 5. Comparison of copy number alteration profiles in cancer genomes between Fe-NTA-induced rat RCCs and human tumors.** (A) The color plot represents a similarity matrix across the rat RCCs and various human cancers. rRCC, rat renal cell carcinoma; hPDAC, human pancreatic ductal adenocarcinoma; hTALL, human T-cell acute lymphoblastic leukemia; hGBM, human glioblastoma multiforme; hRCC, human renal cell carcinoma. (B) Numerical summary of the similarity matrix. The number in each square indicates an average value of similarity index (defined between −1000 and 1,000). Refer to the Materials and Methods section for details.
doi:10.1371/journal.pone.0043403.g005

well as ionic homeostasis maintenance, regeneration from the remaining tubular cells is intensely promoted. Under chronic oxidative stress by repeated Fe-NTA administrations, this degeneration and regeneration process would continue for months to years, increasing the risk of mitotic events simultaneously with the repair of oxidative DNA damage. We believe that this oxidative stress causes abnormal DNA replication and chromosomal missegregation, which leads to the emergence of aneuploid cells. Surprisingly, this series of events appear to occur in months, leading to a high incidence (~90%) of RCC in rats within two years. Aneuploid cells usually exhibit phenotypes consistent with increased energy need and proteotoxic stress. However, aneuploidy can promote tumorigenesis under the following two hypothetical mechanisms: 1) aneuploidy may cause a proliferative advantage through loss of G1/S transition control under conditions in which normal euploid cells do not divide [39] and 2) aneuploidy can advance tumorigenesis by promoting genomic instability, hence increasing the evolvability of tumors [40]. The frequent deletion of the *Cdkn2a/2b* locus is observed in the rat peritoneal mesothelioma, an iron overload-associated tumor [41], induced either by another iron compound (ferric saccharate) [42] or by asbestos [43]. These common traits of animal models strongly suggest that *Cdkn2a/2b* is a principal target in iron-mediated carcinogenesis. The same genetic alteration is observed in rat mesothelioma induced by multi-walled carbon nanotubes [44], in which iron involvement is not yet established. We would like to add here for the biological significance of our results that homozygous deletion of *CDKN2A/2B* is frequently observed in human mesothelioma associated with asbestos exposure [**45,46**].

Some of the tumors with a remaining *Cdkn2a/2b* allele showed extremely high expression levels of two products from this locus, *p16/Ink4a* and *p19/Arf*. This is a currently debated issue in human cancer [47]. There is considerable evidence that several neoplasms exhibit significant p16 levels in cytoplasm [48]. This can be an unsuccessful attempt to stop cell proliferation in the case of downstream *Rb* dysregulation [49] or may represent an alternative mechanism for modulating unidentified pathways. Our data exhibit ~50% hemizygous deletion of *Rb*. This requires further clarification with epigenetic analysis.

The second feature determined using frequency plot analysis was a high incidence of amplification along a limited chromosomal region toward the centromere of chromosome 4, pointing to the *Met* locus. A region spanning 80 Mb from the centromere of rat chromosome 4 is syntenic to human chromosome 7. Various human cancers, such as glioblastoma [50] and non-small cell lung cancer [51], are reported to harbor amplifications in chromosome 7. Tyrosine kinase MET is a receptor for a hepatocyte growth factor and is situated upstream of *ras* in the signal transduction pathway, thus serving as an advantage for cell proliferation [52]. Therefore, it is conceivable that tumor size was proportionally associated with the *Met* expression level (**Figs. 3C and 4A**). Because we dissect the animal as soon as we recognize the tumor, we believe that large-sized tumors are more aggressive in nature. It is of note that tumor size was also related to the genome alteration pattern (**Fig. 4B**), and was associated with amplification and overexpression of *Zbtb38* located on chromosome 8 (**Fig. S4**). ZBTB38 (CIBZ) represses the transcription of methylated templates [53], thus presumably regulating epigenetic mechanisms. Down-regulation of ZBTB38, a novel substrate of caspase-3, induces apoptosis [54] and this gene is localized in a prostate cancer susceptibility locus [54]. These results confirm the possibility of tumor classification using array-based CGH. *Met* arose evolutionally late and is unique to mammals [52]; it could thereby be associated with the unique amplification in the whole genome.

Genomic amplification is hypothesized to occur via the breakage-fusion-bridge cycle [55,56,57]. A Fenton reaction causes double-stranded DNA breakage [10]. Our results revealed that these amplifications consisted of a mixture of wide-range low-level amplifications and fragmented, narrow high-level amplifications

(**Fig. 3A**). This suggests a mechanism of positive feedback for amplification, starting from wide-range low-level amplification. We suspect an involvement of double-minutes, and a presence of susceptible genomic loci. This hypothesis requires further study. It was interesting that two tumor suppressive genes, *Cav1* [58,59] and *ST7* [60], surrounded the *Met* locus (**Fig. 3B**). This may be the reason why the *Met* locus was a denominator for the rat RCCs. Whole exome or genome sequencing may further reveal new findings regarding point mutation and chromosomal translocation.

Finally, we compared the present rat results with corresponding human tumors by transforming data based on chromosomal synteny (**Figs. 5A and 5B**). It was expected that the genomic alteration of Fe-NTA-induced rat RCC was most similar to human RCC presumably because target cells are the same. However, surprisingly, human mesothelioma was the second most similar. It is now established that most human mesothelioma results from exposure to asbestos, and the primary pathogenic process involved is iron overload [**8,61**]. The same mesodermal origin of renal tubular cells and mesothelial cells may cause the similarity of the array-based CGH profiles. Endodermal tumor, such as pancreatic ductal adenocarcinoma (PDAC), and ectodermal tumor, such as glioblastoma multiforme (GBM), exhibited a significant difference in genomic profiles.

In conclusion, we showed that repeated Fenton reactions in wild-type rats induced cancer that recapitulated genomic alterations similar to those in human cancers, suggesting the involvement of oxidative stress as a major factor in human carcinogenesis. In this renal carcinogenesis model, preferred alterations were deletion; *Cdkn2A/2B* deletion and *Met* amplification were the major target gene modifications. A comparative interspecies analysis would contribute to identifying candidate carcinogenic agents.

## Materials and Methods

### Fe-NTA-induced Renal Cell Carcinoma Model

Fe-NTA-induced carcinogenesis experiments were performed using male F1 hybrid rats that were a cross between Fischer 344 and Brown-Norway inbred strains (Charles River, Yokohama, Japan) as previously described [62]. Thirteen RCC cases were used in this study, and the histological grade of the tumor was determined according to the modified World Health Organization classification as we previously described [62]. The details are summarized in **Table 1**. The animal experiment committees of the Graduate School of Medicine, Kyoto University Graduate School of Medicine and Nagoya University Graduate School of Medicine approved this study. FRCC001 and FRCC562 cell lines were established from primary Fe-NTA-induced RCCs as previously described [63].

### Array-based Comparative Genomic Hybridization

Genomic DNA from the tumors and the cell lines was isolated with DNeasy (Qiagen, Valencia, CA). Array-based CGH was performed with an Agilent 185 K rat genome CGH microarray (Agilent Technologies, Santa Clara, CA) as previously described [64]. Thirteen primary tumors and two cell lines of Fe-NTA induced RCCs were analyzed using reference DNA extracted from a normal kidney of a rat from Brown-Norway imbred strain. One RCC sample of a female Eker rat [32] was analyzed using reference DNA extracted from a normal kidney of a male Eker rat. Two additional RCC samples of male Eker rats were analyzed with Rat Genome CGH Microarray 105A (G4436A; Agilent Technologies), using reference DNA extracted from a normal liver of another male Eker rat. The normalized array-based CGH data were processed to generate a segmented profile by circular binary segmentation (CBS) [65] with an altered significance level (alpha = 0.0001). The procedure of data processing for copy number estimation is detailed in **Methods S1**.

### Quantitative RT-PCR Analysis

Total RNA was isolated using Isogen reagents (Nippon Gene Co. Ltd., Tokyo, Japan) according to the manufacturer's protocol. cDNA was synthesized using an RNA PCR kit ver. 3.0 (Takara Bio, Shiga, Japan) with random primers. A Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen, Carlsbad, CA) and a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) were used for quantitative real-time PCR analysis. Rat β-actin was used as an internal control. The primers used were as follows: $p16^{Ink4a}$, 5′-aaacgccccgaacactttc-3′ and 5′-gttcgaatctgcaccatagga-3′; $p19^{Arf}$, 5′-accccaagtgagggttttct-3′ and 5′-agagctgccactttgacgtt-3′; $p15^{Ink4b}$, 5′-tccacaggctagagggaaaa-3′ and 5′-gtgcaggtgactccttggtt-3′; *Met*, 5′-ttaagcgagagcacgacaaat-3′ and 5′-ccacataggaaaacgcactgt-3′; *Zbtb38*, 5′-gtagctgctgctccaaatcc-3′ and 5′-cctgttgagggtggtgaact-3′; β-actin, 5′-tgtgttgtccctgtatgcctctg-3′ and 5′-atagatgggcacagtgtgggtg-3′.

### Human Data

We used human array-based CGH data of pancreatic ductal adenocarcinoma (hPDAC) [28], T-cell acute lymphoblastic leukemia (hTALL) [28], glioblastoma (hGBM) [66], mesothelioma cell line (hMM) [67] and renal cell carcinoma (hRCC) [56]. The Agilent CGH-array data of the former four types of human cancer were obtained from NCBI's Gene Expression Omnibus (GEO) website (http://www.ncbi.nlm.nih.gov/geo/). The GEO accession numbers for the data sets are GSE7599 (hPDAC), GSE7603 (hTALL), GSE9177 (hGBM) and GSE22237 (hMM). The human RCC data was obtained through analyses with BAC microarrays (4,361 clones) [68]. We defined similarity index between the two array-based CGH profiles as follows. First, we calculated the correlation coefficient with log2 ratios of the estimated copy number over the inferred cancer ploidy for the genomic positions corresponding to all of the Agilent 44 K human CGH microarray probes. Then, we multiplied the value by $1 \times 10^3$ after changing the absolute value into its square root.

## Supporting Information

**Figure S1 Array-CGH profiles from all the RCCs examined.** Red lines show log2 ratios of estimated copy number over inferred cancer ploidy versus genomic position for all the CGH microarray probes.
(PDF)

**Figure S2 Distribution of log2 ratio values of estimated copy number for all the probes in all the microarrays performed.**
(PDF)

**Figure S3 Example of global expression changes in line with genomic alteration.** Differences in genome and transcriptome are analyzed between two RCCs, FB7-7 having wide-range amplification on chromosome 4 versus FB28-7 having no substantial genomic alteration on chromosome 4. (A) Sky blue circle plot indicates ratio of estimated copy numbers based on array-based CGH (FB7-7 *vs* FB28-7). Red circle plot indicates ratio of normalized signals on Affymetrix expression microarray (FB7-7 *vs* FB28-7). (B) Expression ratio values are averaged along the chromosome. Here, red circle plot indicates average value in 2-Mb windows.
(PDF)

**Figure S4 *Zbtb38* mRNA expression is demonstrably associated with its chromosomal copy number.** (A) Array-CGH profiles of two RCC tumors harboring amplification over *Zbtb38* locus on chromosome 8. (B) Expression analysis of *Zbtb38* on 13 RCC tumors by real-time PCR. The values of the *y*-axis indicate the relative mRNA expression level compared to an average of those in normal kidneys of three control rats.
(PDF)

**Figure S5 Array-CGH profiles of three hereditary (Eker rat) renal tumors.** Red lines show log2 ratios of estimated copy number over inferred cancer ploidy versus genomic position for all the CGH microarray probes.
(PDF)

**Table S1 List of genes completely deleted in more than two RCC tumors.**
(XLS)

**Table S2 List of genes amplified in more than two RCC tumors.**
(XLS)

**Methods S1 Supplementary methods.**
(DOC)

## Author Contributions

Conceived and designed the experiments: SA ST. Performed the experiments: SA YY HO YTL MI KA MO LJ H. Nagai HM EA. Analyzed the data: SA YO TT ST. Contributed reagents/materials/analysis tools: YS YK OH H. Nakagama. Wrote the paper: SA ST. Designed the software used in analysis: SA.

## References


1. Weinberg RA (2007) The biology of cancer. New York: Garland Science, Tailor & Francis Group, LLC.
2. Klijn C, Holstege H, de Ridder J, Liu X, Reinders M, et al. (2008) Identification of cancer genes using a statistical framework for multiexperiment analysis of nondiscretized array CGH data. Nucleic Acids Res 36: e13.
3. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, et al. (2008) An integrated genomic analysis of human glioblastoma multiforme. Science 321: 1807–1812.
4. Pfeifer GP, Hainaut P (2011) Next-generation sequencing: emerging lessons on the origins of human cancer. Curr Opin Oncol 23: 62–68.
5. Toyokuni S (1999) Reactive oxygen species-induced molecular damage and its applicaton in pathology. PatholInt 49: 91–102.
6. Halliwell B, Gutteridge JMC (2007) Free radicals in biology and medicine. New York: Oxford University Press.
7. Toyokuni S (1996) Iron-induced carcinogenesis: the role of redox regulation. Free Radic Biol Med 20: 553–566.
8. Toyokuni S (2009) Role of iron in carcinogenesis: Cancer as a ferrotoxic disease. Cancer Sci 100: 9–16.
9. Fenton HJH (1894) Oxidation of tartaric acid in presence of iron. J Chem Soc 65: 899–910.
10. Toyokuni S, Sagripanti JL (1992) Iron-mediated DNA damage: sensitive detection of DNA strand breakage catalyzed by iron. J Inorg Biochem 47: 241–248.
11. Ebina Y, Okada S, Hamazaki S, Ogino F, Li JL, et al. (1986) Nephrotoxicity and renal cell carcinoma after use of iron- and aluminum- nitrilotriacetate complexes in rats. J Natl Cancer Inst 76: 107–113.
12. Li JL, Okada S, Hamazaki S, Ebina Y, Midorikawa O (1987) Subacute nephrotoxicity and induction of renal cell carcinoma in mice treated with ferric nitrilotriacetate. Cancer Res 47: 1867–1869.
13. Toyokuni S, Mori T, Dizdaroglu M (1994) DNA base modifications in renal chromatin of Wistar rats treated with a renal carcinogen, ferric nitrilotriacetate. Int J Cancer 57: 123–128.
14. Toyokuni S, Tanaka T, Hattori Y, Nishiyama Y, Ochi H, et al. (1997) Quantitative immunohistochemical determination of 8-hydroxy-2′-deoxyguanosine by a monoclonal antibody N45.1: its application to ferric nitrilotriacetate-induced renal carcinogenesis model. Lab Invest 76: 365–374.
15. Toyokuni S, Uchida K, Okamoto K, Hattori-Nakakuki Y, Hiai H, et al. (1994) Formation of 4-hydroxy-2-nonenal-modified proteins in the renal proximal tubules of rats treated with a renal carcinogen, ferric nitrilotriacetate. Proc Natl Acad Sci USA 91: 2616–2620.
16. Toyokuni S, Luo XP, Tanaka T, Uchida K, Hiai H, et al. (1997) Induction of a wide range of $C_{2-12}$ aldehydes and $C_{7-12}$ acyloins in the kidney of Wistar rats after treatment with a renal carcinogen, ferric nitrilotriacetate. Free Radic Biol Med 22: 1019–1027.
17. Gutteridge J, Rowley D, Halliwell B (1981) Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts. Detection of 'free' iron in biological systems by using bleomycin-dependent degradation of DNA. Biochem J 199: p263–265.
18. Sasaki K, Ikuta K, Tanaka H, Ohtake T, Torimoto Y, et al. (2011) Improved quantification for non-transferrin-bound iron measurement using high-performance liquid chromatography by reducing iron contamination. Mol Med Report 4: 913–918.
19. Tanaka T, Iwasa Y, Kondo S, Hiai H, Toyokuni S (1999) High incidence of allelic loss on chromosome 5 and inactivation of $p15^{INK4B}$ and $p16^{INK4A}$ tumor suppressor genes in oxystress-induced renal cell carcinoma of rats. Oncogene 18: 3793–3797.
20. Toyokuni S, Akatsuka S (2006) What has been learned from the studies of oxidative stress-induced carcinogenesis: Proposal of the concept of oxygenomics. J Clin Biochem Nutr 39: 3–10.
21. Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, et al. (1992) Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science 258: 818–821.
22. Macconaill LE, Van Hummelen P, Meyerson M, Hahn WC (2011) Clinical implementation of comprehensive strategies to characterize cancer genomes: opportunities and challenges. Cancer Discov 1: 297.
23. Negrini S, Gorgoulis VG, Halazonetis TD (2011) Genomic instability–an evolving hallmark of cancer. Nat Rev Mol Cell Biol 11: 220–228.
24. Adamovic T, McAllister D, Guryev V, Wang X, Andrae JW, et al. (2009) Microalterations of inherently unstable genomic regions in rat mammary carcinomas as revealed by long oligonucleotide array-based comparative genomic hybridization. Cancer Res 69: 5159–5167.
25. Fernia AP, Luceri C, Toti S, Giannini A, Dolara P, et al. (2010) Gene expression profile and genomic alterations in colonic tumours induced by 1,2-dimethylhydrazine (DMH) in rats. BMC Cancer 10: 194.
26. Takabatake T, Fujikawa K, Tanaka S, Hirouchi T, Nakamura M, et al. (2006) Array-CGH analyses of murine malignant lymphomas: genomic clues to understanding the effects of chronic exposure to low-dose-rate gamma rays on lymphomagenesis. Radiat Res 166: 61–72.
27. Herzog CR, Desai D, Amin S (2006) Array CGH analysis reveals chromosomal aberrations in mouse lung adenocarcinomas induced by the human lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Biochem Biophys Res Commun 341: 856–863.
28. Maser R, Choudhury B, Campbell P, Feng B, Wong K, et al. (2007) Chromosomally unstable mouse tumours have genomic alterations similar to diverse human cancers. Nature 447: 966–971.
29. Loeb LA, Bielas JH, Beckman RA (2008) Cancers exhibit a mutator phenotype: clinical implications. Cancer Res 68: 3551–3557.
30. Hisada M, Garber JE, Fung CY, Fraumeni JF, Li FP (1998) Multiple primary cancers in families with Li-Fraumeni syndrome. J Natl Cancer Inst 90: 606–611.
31. Gruis NA, van der Velden PA, Sandkuijl LA, Prins DE, Weaver-Feldhaus J, et al. (1995) Homozygotes for CDKN2 (p16) germline mutation in Dutch familial melanoma kindreds. Nat Genet 10: 351–353.
32. Kobayashi T, Hirayama Y, Kobayashi E, Kubo Y, Hino O (1995) A germline insertion in the tuberous sclerosis (Tsc2) gene give rise to the Eker rat model of dominantly inherited cancer. Nature Genet 9: 70–74.
33. Hino O (2004) Multistep renal carcinogenesis in the Eker (Tsc 2 gene mutant) rat model. Curr Mol Med 4: 807–811.
34. Yeung RS (2004) Lessons from the Eker rat model: from cage to bedside. Curr Mol Med 4: 799–806.
35. Huang X (2003) Iron overload and its association with cancer risk in humans: evidence for iron as a carcinogenic metal. Mutat Res 533: 153–171.
36. Hiroyasu M, Ozeki M, Kohda H, Echizenya M, Tanaka T, et al. (2002) Specific allelic loss of p16 (INK4A) tumor suppressor gene after weeks of iron-mediated oxidative damage during rat renal carcinogenesis. Am J Pathol 160: 419–424.
37. Toyokuni S, Okada S, Hamazaki S, Minamiyama Y, Yamada Y, et al. (1990) Combined histochemical and biochemical analysis of sex hormone dependence of ferric nitrilotriacetate-induced renal lipid peroxidation in ddY mice. Cancer Res 50: 5574–5580.
38. Zhang D, Okada S, Yu Y, Zheng P, Yamaguchi R, et al. (1997) Vitamin E inhibits apoptosis, DNA modification, and cancer incidence induced by iron-mediated peroxidation in Wistar rat kidney. Cancer Res 57: 2410–2414.
39. Torres EM, Williams BR, Tang YC, Amon A (2010) Thoughts on aneuploidy. Cold Spring Harb Symp Quant Biol 75: 445–451.
40. Torres EM, Williams BR, Amon A (2008) Aneuploidy: cells losing their balance. Genetics 179: 737–746.
41. Toyokuni S (2009) Mechanisms of asbestos-induced carcinogenesis. Nagoya J Med Sci 71: 1–10.

42. Hu Q, Akatsuka S, Yamashita Y, Ohara H, Nagai H, et al. (2010) Homozygous deletion of *CDKN2A/2B* is a hallmark of iron-induced high-grade rat mesothelioma. Lab Invest 90: 360–373.
43. Jean D, Thomas E, Manie E, Renier A, de Reynies A, et al. (2011) Syntenic relationships between genomic profiles of fiber-induced murine and human malignant mesothelioma. Am J Pathol 178: 881–894.
44. Nagai H, Okazaki Y, Hwu C, Misawa N, Yamashita Y, et al. (2011) Diameter of multi-walled carbon nanotubes is a critical factor in mesothelial injury and subsequent carcinogenesis. Proc Natl Acad Sci USA.
45. Cheng J, Jhanwar S, Klein W, Bell D, Lee W, et al. (1994) p16 alterations and deletion mapping of 9p21-p22 in malignant mesothelioma. Cancer Res 54: 5547–5551.
46. Xio S, Li D, Vijg J, Sugarbaker D, Corson J, et al. (1995) Codeletion of *p15* and *p16* in primary malignant mesothelioma. Oncogene 11: p511–515.
47. Romagosa C, Simonetti S, Lopez-Vicente L, Mazo A, Lleonart ME, et al. (2011) p16(Ink4a) overexpression in cancer: a tumor suppressor gene associated with senescence and high-grade tumors. Oncogene 30: 2087–2097.
48. Evangelou K, Bramis J, Peros I, Zacharatos P, Dasiou-Plakida D, et al. (2004) Electron microscopy evidence that cytoplasmic localization of the p16(INK4A) "nuclear" cyclin-dependent kinase inhibitor (CKI) in tumor cells is specific and not an artifact. A study in non-small cell lung carcinomas. Biotech Histochem 79: 5–10.
49. Reuschenbach M, Waterboer T, Wallin KL, Einenkel J, Dillner J, et al. (2008) Characterization of humoral immune responses against p16, p53, HPV16 E6 and HPV16 E7 in patients with HPV-associated cancers. Int J Cancer 123: 2626–2631.
50. Wullich B, Sattler HP, Fischer U, Meese E (1994) Two independent amplification events on chromosome 7 in glioma: amplification of the epidermal growth factor receptor gene and amplification of the oncogene MET. Anticancer Res 14: 577–579.
51. Campbell JM, Lockwood WW, Buys TP, Chari R, Coe BP, et al. (2008) Integrative genomic and gene expression analysis of chromosome 7 identified novel oncogene loci in non-small cell lung cancer. Genome 51: 1032–1039.
52. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF (2003) Met, metastasis, motility and more. Nat Rev Mol Cell Biol 4: 915–925.
53. Filion GJ, Zhenilo S, Salozhin S, Yamada D, Prokhortchouk E, et al. (2006) A family of human zinc finger proteins that bind methylated DNA and repress transcription. Mol Cell Biol 26: 169–181.
54. Oikawa Y, Matsuda E, Nishii T, Ishida Y, Kawaichi M (2008) Down-regulation of CIBZ, a novel substrate of caspase-3, induces apoptosis. J Biol Chem 283: 14242–14247.
55. Hellman A, Zlotorynski E, Scherer SW, Cheung J, Vincent JB, et al. (2002) A role for common fragile site induction in amplification of human oncogenes. Cancer Cell 1: 89–97.
56. Albertson DG (2006) Gene amplification in cancer. Trends Genet 22: 447–455.
57. Martinez AC, van Wely KH (2010) Are aneuploidy and chromosome breakage caused by a CINgle mechanism? Cell Cycle 9: 2275–2280.
58. Wiechen K, Diatchenko L, Agoulnik A, Scharff KM, Schober H, et al. (2001) Caveolin-1 is down-regulated in human ovarian carcinoma and acts as a candidate tumor suppressor gene. Am J Pathol 159: 1635–1643.
59. Wiechen K, Sers C, Agoulnik A, Arlt K, Dietel M, et al. (2001) Down-regulation of caveolin-1, a candidate tumor suppressor gene, in sarcomas. Am J Pathol 158: 833–839.
60. Pal S, Vishwanath SN, Erdjument-Bromage H, Tempst P, Sif S (2004) Human SWI/SNF-associated PRMT5 methylates histone H3 arginine 8 and negatively regulates expression of ST7 and NM23 tumor suppressor genes. Mol Cell Biol 24: 9630–9645.
61. Nagai H, Ishihara T, Lee WH, Ohara H, Okazaki Y, et al. (2011) Asbestos surface provides a niche for oxidative modification. Cancer Sci 102: 2118–2125.
62. Nishiyama Y, Suwa H, Okamoto K, Fukumoto M, Hiai H, et al. (1995) Low incidence of point mutations in *H-, K-* and *N-ras* oncogenes and *p53* tumor suppressor gene in renal cell carcinoma and peritoneal mesothelioma of Wistar rats induced by ferric nitrilotriacetate. Jpn J Cancer Res 86: 1150–1158.
63. Tanaka T, Akatsuka S, Ozeki M, Shirase T, Hiai H, et al. (2004) Redox regulation of annexin 2 and its implications for oxidative stess-induced renal carcinogenesis and metastasis. Oncogene 23: 3980–3989.
64. Liu Y-T, Shang D-G, Akatsuka S, Ohara H, Dutta KK, et al. (2007) Chronic oxidative stress causes amplification and overexpresson of *ptprz1* protein tyrosine phosphatase to activate β-catenin pathway. Am J Pathol 171: 1978–1988.
65. Olshen AB, Venkatraman ES, Lucito R, Wigler M (2004) Circular binary segmentation for the analysis of array-based DNA copy number data. Biostatistics 5: 557–572.
66. Wiedemeyer R, Brennan C, Heffernan TP, Xiao Y, Mahoney J, et al. (2008) Feedback circuit among INK4 tumor suppressors constrains human glioblastoma development. Cancer Cell 13: 355–364.
67. Murakami H, Mizuno T, Taniguchi T, Fujii M, Ishiguro F, et al. (2011) LATS2 is a tumor suppressor gene of malignant mesothelioma. Cancer Res 71: 873–883.
68. Arai E, Ushijima S, Tsuda H, Fujimoto H, Hosoda F, et al. (2008) Genetic clustering of clear cell renal cell carcinoma based on array-comparative genomic hybridization: its association with DNA methylation alteration and patient outcome. Clin Cancer Res 14: 5531–5539.

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i22.2745

*World J Gastroenterol* 2012 June 14; 18(22): 2745-2755
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


GUIDELINES FOR BASIC SCIENCE

# Interrelationship between microsatellite instability and microRNA in gastrointestinal cancer

Hiroyuki Yamamoto, Yasushi Adachi, Hiroaki Taniguchi, Hiroaki Kunimoto, Katsuhiko Nosho, Hiromu Suzuki, Yasuhisa Shinomura

Hiroyuki Yamamoto, Yasushi Adachi, Hiroaki Kunimoto, Katsuhiko Nosho, Hiromu Suzuki, Yasuhisa Shinomura, First Department of Internal Medicine, Sapporo Medical University School of Medicine, Sapporo 060-8543, Japan
Hiroaki Taniguchi, Division of Cancer Cell Research, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan
Hiromu Suzuki, Department of Molecular Biology, Sapporo Medical University School of Medicine, Sapporo 060-8556, Japan
Author contributions: Yamamoto H conceived the topic, reviewed the literature and prepared the manuscript; Adachi Y, Taniguchi H, Kunimoto H, Nosho K and Suzuki H reviewed and analyzed the literature; and Shinomura Y provided intellectual support.
Supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan
Correspondence to: Hiroyuki Yamamoto, MD, FJSIM, PhD, First Department of Internal Medicine, Sapporo Medical University School of Medicine, S1W16 Chuo-ku, Sapporo 060-8543, Japan. h-yama@sapmed.ac.jp
Telephone: +81-11-6112111 Fax: +81-11-6112282
Received: September 28, 2011 Revised: March 2, 2012
Accepted: March 9, 2012
Published online: June 14, 2012

## Abstract

There is an increasing understanding of the roles that microsatellite instability (MSI) plays in Lynch syndrome (by mutations) and sporadic (by mainly epigenetic changes) gastrointestinal (GI) and other cancers. Deficient DNA mismatch repair (MMR) results in the strong mutator phenotype known as MSI, which is the hallmark of cancers arising within Lynch syndrome. MSI is characterized by length alterations within simple repeated sequences called microsatellites. Lynch syndrome occurs primarily because of germline mutations in one of the MMR genes, mainly *MLH1* or *MSH2*, less frequently *MSH6*, and rarely *PMS2*. MSI is also observed in about 15% of sporadic colorectal, gastric, and endometrial cancers and in lower frequencies in a minority of other cancers where it is often associated with the hypermethylation of the *MLH1* gene. miRNAs are small noncoding RNAs that regulate gene expression at the posttranscriptional level and are critical in many biological processes and cellular pathways. There is accumulating evidence to support the notion that the interrelationship between MSI and miRNA plays a key role in the pathogenesis of GI cancer. As a possible new mechanism underlying MSI, overexpression of *miR-155* has been shown to downregulate expression of MLH1, MSH2, and MSH6. Thus, a subset of MSI-positive (MSI+) cancers without known MMR defects may result from *miR-155* overexpression. Target genes of frameshift mutation for MSI are involved in various cellular functions, such as DNA repair, cell signaling, and apoptosis. A novel class of target genes that included not only epigenetic modifier genes, such as *HDAC2*, but also miRNA processing machinery genes, including *TARBP2* and *XPO5*, were found to be mutated in MSI+ GI cancers. Thus, a subset of MSI+ colorectal cancers (CRCs) has been proposed to exhibit a mutated miRNA machinery phenotype. Genetic, epigenetic, and transcriptomic differences exist between MSI+ and MSI− cancers. Molecular signatures of miRNA expression apparently have the potential to distinguish between MSI+ and MSI− CRCs. In this review, we summarize recent advances in the MSI pathogenesis of GI cancer, with the focus on its relationship with miRNA as well as on the potential to use MSI and related alterations as biomarkers and novel therapeutic targets.



**Key words:** Microsatellite instability; MicroRNA; DNA mismatch repair; Frameshift mutation; MicroRNA processing

**Peer reviewers:** Dr. John Souglakos, Department of Medical Oncology, University Hospital of Heraklion and Laboratory of Cancer Biology, 71110 Heraklion, Greece; Dr. Jose Perea, De-

partment of Surgery, 12 De Octubre University Hospital, Rosas De Aravaca 82A, 28023 Madrid, Spain

Yamamoto H, Adachi Y, Taniguchi H, Kunimoto H, Nosho K, Suzuki H, Shinomura Y. Interrelationship between microsatellite instability and microRNA in gastrointestinal cancer. *World J Gastroenterol* 2012; 18(22): 2745-2755 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i22/2745.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i22.2745

## INTRODUCTION

A type of genetic instability characterized by length alterations within simple repeated microsatellite sequences, termed microsatellite instability (MSI), occurs in a majority of patients with Lynch syndrome (also known as hereditary nonpolyposis colorectal cancer) by mutations and in a subset of sporadic gastrointestinal (GI) and other cancers by mainly epigenetic changes[1-10]. Genetic and epigenetic inactivation of DNA mismatch repair (*MMR*) genes results in the mutator phenotype, mutations in cancer-related genes, and cancer development (Figure 1). MSI underlies a distinctive carcinogenic pathway because MSI-positive (MSI+) cancers exhibit many differences in clinical, pathological, and molecular characteristics relative to MSI-negative (MSI-) cancers irrespective of their hereditary or sporadic origins. The differences in genotype can be explained because deficient MMR leads to a strong mutator phenotype with a very specific mutation spectrum. MSI accumulates frameshift mutations in repeated sequences located in coding regions of target tumor suppressor genes. The peculiar genotype of MSI+ cancers also includes specific patterns of gene regulation. MSI+ GI cancers often show an aberrant epigenetic pattern such as hypermethylation of various genes, including the key *MMR* gene, *MLH1*. The differences in genotype and phenotype between MSI+ and MSI- GI cancers are likely to be causally linked to their differences in biological and clinical features. Diagnostic characterization of the MSI status thus has implications in basic and clinical oncology. MiRNAs are small RNA molecules that regulate gene expression at the posttranscriptional level and are critical for many cellular functions[11-20]. There is accumulating evidence to support the notion that the interrelationship between MSI and miRNA plays a key role in the pathogenesis of GI cancer.

## MSI BY THE OVEREXPRESSION OF MIR-155 OR MIR-21

Various pathogenic events, including germline and somatic mutations, promoter methylation, and reduced histone acetylation[21], lead to inactivation of core MMR proteins. A vast majority of MSI+ cancers can be explained by mutation and/or epigenetic inactivation of the core MMR proteins[22]. The etiologies of the remaining MSI+ cancers remain poorly understood. In an unselected series of 1066 colorectal cancer (CRC) patients, 135 (13%) were MSI+[22]. Of these, 23 (6%) had germline mutations in one of the *MMR* gene, and 106 (79%) showed methylation of the *MLH1* promoter. Approximately 5% of these MSI+ cancers displayed loss of expression of at least one of the core MMR proteins without a well-defined genetic or epigenetic cause[22].

Vareli *et al*[23] have shown that overexpression of *miR-155* significantly downregulates the core MMR proteins; namely, MLH1, MSH2, and, MSH6 in CRC cell lines, thus inducing an MSI. The downregulation of MLH1 and MSH2 proteins by *miR-155* lead to destabilization of the respective heterodimeric complex proteins and a mutator phenotype[24]. An inverse correlation between *miR-155* overexpression and the expression of MLH1 and MSH2 was further demonstrated in human CRC tissues. Most MSI+ cancers without a known cause of MMR inactivation show *miR-155* overexpression. However, not all CRCs with increased miR-155 expression were MSI+. It is also possible that miR-155 affects other related DNA repair proteins, thus enhancing the phenotypic effect of MMR defects. Although further confirmation is required, the results suggest that *miR-155* overexpression is an additional mechanism underlying the development of MSI in cancer (Figure 2)[23].

The reduced expression of a single allele of the adenomatous polyposis coli and transforming growth factor (*TGF*)-β receptor I gene has been linked to CRC[25,26]. Thus, incomplete repression of MMR proteins by *miR-155* is not unique to tumor suppressor genes in cancer. Recently, miRNAs have been suggested to act as transactivating elements involved in allele and gene expression regulation[27,28]. These results support the notion that miRNAs play a role in the non-Mendelian regulation of *MMR* genes.

*miR-21* is overexpressed in various types of human cancers, including CRC[29]. Valeri *et al*[30] reported that *miR-21* directly targets the 3' untranslated region (UTR) of *MSH2* and *MSH6* mRNA, resulting in downregulation of protein expression. The inverse correlation between *miR-21* overexpression and MSH2 expression was shown in CRC tissues. Cells that overexpress *miR-21* showed significantly reduced 5-fluorouracil (5-FU)-induced G2/M damage arrest and apoptosis that is characteristic of defective MMR. Because *miR-21* expression could increase in cell lines continuously exposed to 5-FU[31], cancer cells may develop a secondary resistance to 5-FU through *miR-21* overexpression. Thus, *miR-21*-dependent downregulation of *MSH2-MSH6* may be responsible for both primary and secondary resistance to 5-FU.

## TARGET CANCER-RELATED GENES OF MSI

The instability in cancer-related genes at coding microsatellites causes frameshift mutations and functional inactivation of affected proteins, thereby providing a selective growth advantage to deficient MMR cells[32]. For instance, *TGF-β receptor II* and the pro-apoptotic gene *BAX* are

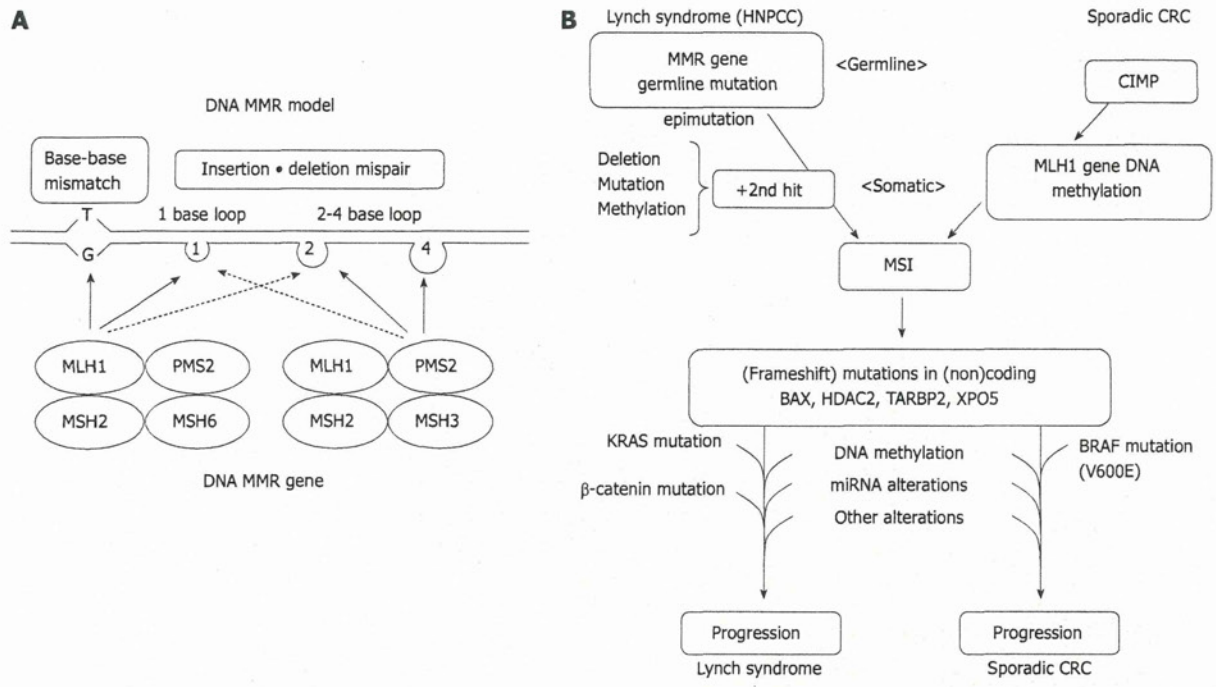


**Figure 1 A model of DNA mismatch repair and molecular pathways for microsatellite instability+ colorectal cancers.** A: A model of the proposed mechanism of mismatch repair (MMR) proteins, illustrating patterns of relevant heterodimerization; B: The models for colorectal cancer (CRC) carcinogenesis are presented in parallel for Lynch syndrome and sporadic cases. HNPCC: Hereditary nonpolyposis colorectal cancer; MSI: Microsatellite instability; CIMP: CpG island methylator phenotype; MSI: Microsatellite instability.

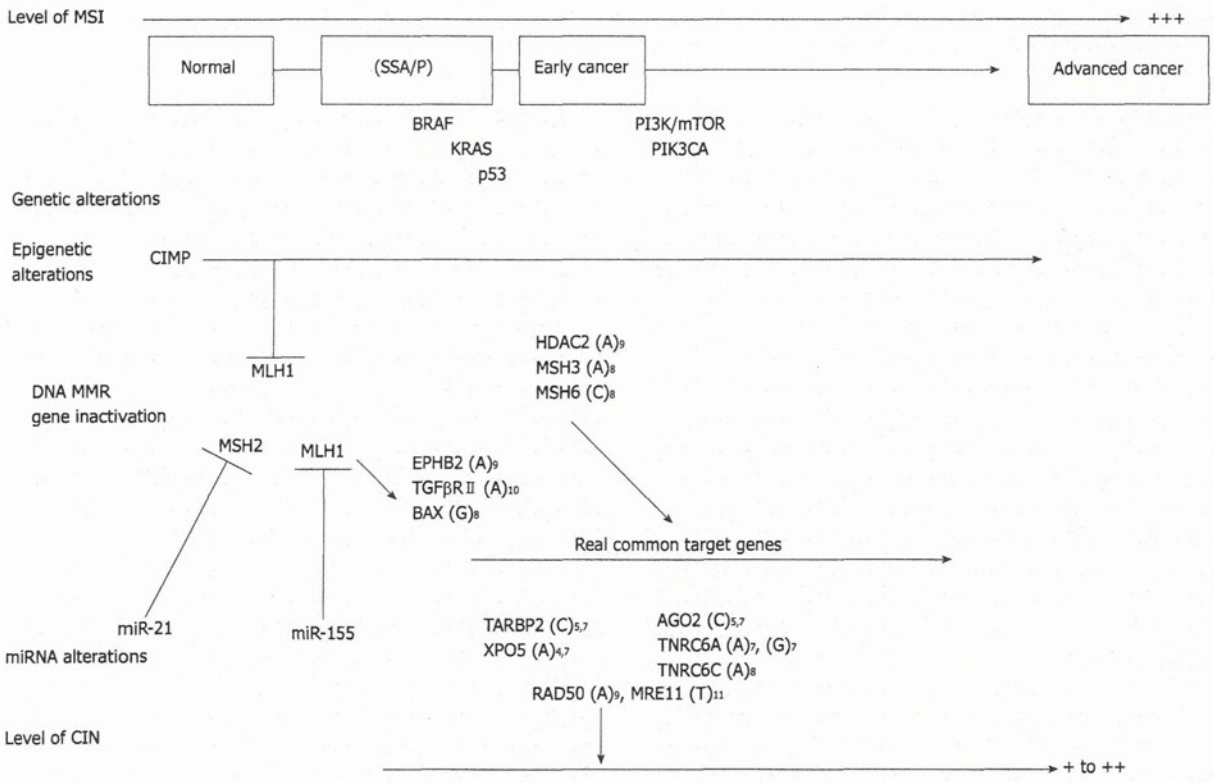


**Figure 2 Cancer progression of sporadic microsatellite instability+ colorectal cancers.** The model for microsatellite instability (MSI)+ colorectal cancer progression is presented based on levels of MSI and chromosomal instability (CIN), and genetic, epigenetic and miRNA alterations. SSA/P: Sessile serrated adenomas/polyps; CIMP: CpG island methylator phenotype; MMR: Mismatch repair.

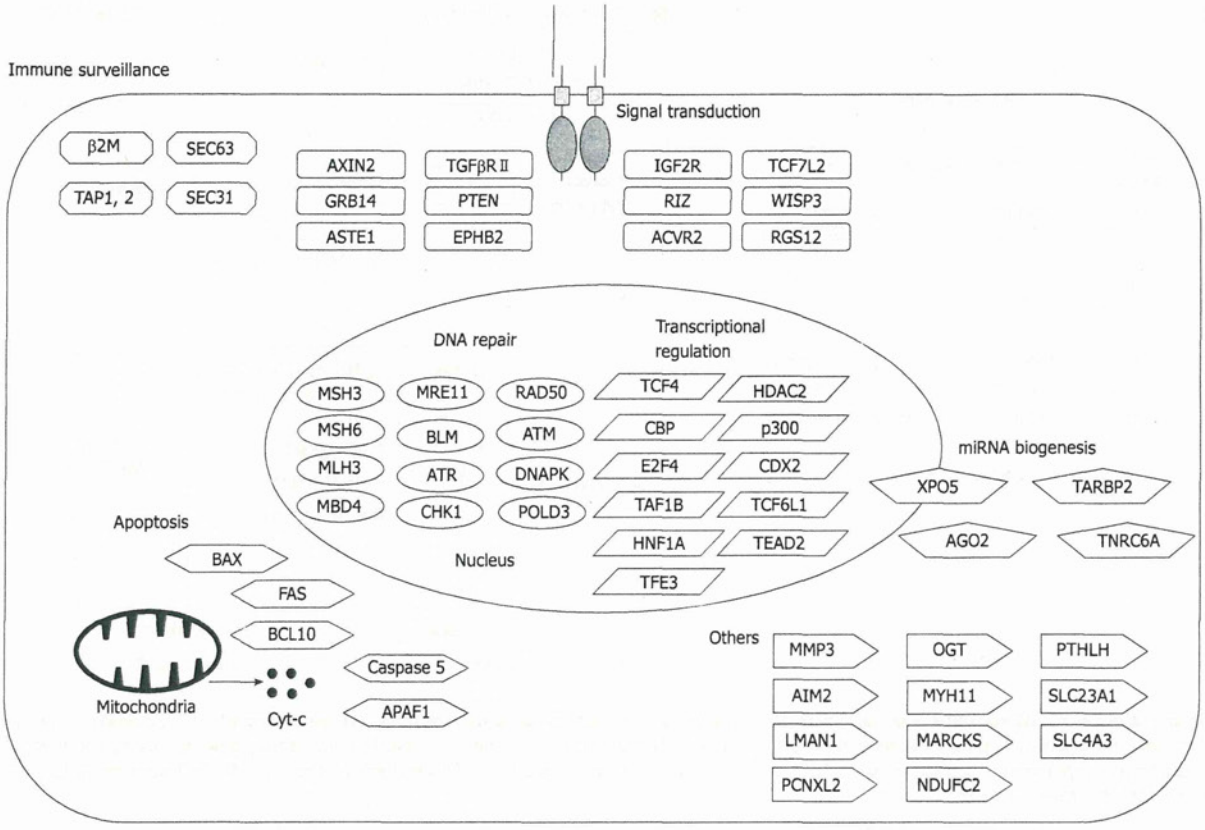


**Figure 3 Representative target genes in microsatellite instability+ gastrointestinal cancers.** A number of cancer-related genes mutated in microsatellite instability+ gastrointestinal cancers have been reported. The relevance of each mutation is not necessarily proven.

frequently inactivated by slippage-induced frameshift mutations in mononucleotide tracts present in their gene coding regions[33,34]. These findings have provided proof for the causative link between MSI and mutations in cancer-related genes, and they were also convincing examples of the differences between the mutator and suppressor pathways for cancer. These genes have also been mutated in cancers in the suppressor pathway, but at decreased frequencies and not by slippage-linked frameshifts[35,36].

A number of cancer-related genes mutated in MSI+ cancers have been reported (Figure 3). Mutations that promote cancer cell growth are assumed to be the driving force during MSI+ carcinogenesis and are designated as real common target genes (Figure 2)[37]. Mutations of microsatellite-harboring genes that do not contribute to carcinogenesis are designated as bystander genes. However, it is not always clear which mutations are "driver mutations" and which are "passenger mutations"[9,38]. The Selective Targets database (SelTarbase) (http://www.seltarbase.org) of human mononucleotide-microsatellite mutations and their potential impact to carcinogenesis and immunology has been developed[39]. The database includes a comprehensive database of all human coding, untranslated, noncoding RNA and intronic mononucleotide repeat tracts and is useful for basic and clinical oncology.

Because MSI+ cancers accumulate many mutations, disruption of cell growth and survival regulation can be accomplished in different cancers by mutations in different genes of the same signaling pathways[40]. Therefore, genes with infrequent and/or monoallelic mutations should not be regarded as irrelevant. Thus, the relevance of microsatellite-specific mutations in MSI+ cancers can be proven only when there is supporting evidence for functionality irrespective of mutation incidence[2].

Target cancer-related genes of MSI+ cancers can be functionally categorized as tumor suppressors and genes involved in DNA repair, cell cycle, cell proliferation, apoptosis and others (Figure 3). Interestingly, every human *MMR* gene except *MLH1* contains a mononucleotide repeat of at least A7[41]. Thus, frameshift mutations of *MSH3* and *MSH6* led to the concept of "the mutator that mutates the other mutator" (Figure 2)[42].

The spectrum of mutations of target genes could affect cancer biology, therapeutic response, and prognosis of patients. Most putative MSI target genes have been proposed mainly on the basis of high mutation frequency detected within their coding regions. However, genes containing microsatellites that are located within noncoding regulatory regions, such as introns, promoters, and 5' and 3' UTRs, could be also mutated in MSI+ cancers. Alterations within untranslated mononucleotide repeat tracts can alter transcription level or transcript stability. It has been suggested that some intronic repeat mutations in genes, such as *ATM*, *MYB*[43], and *MRE11*[44], play a

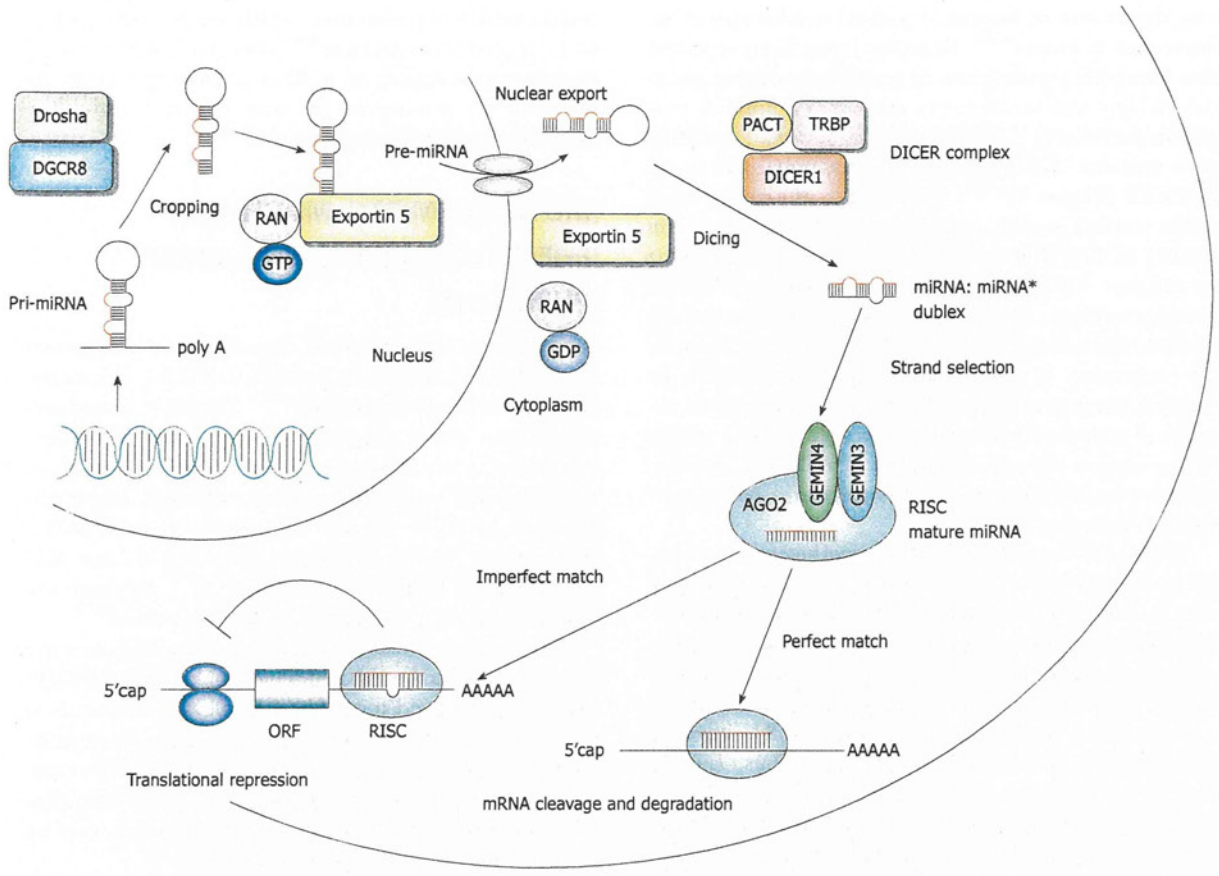


**Figure 4 MiRNA biogenesis and genes mutated in microsatellite instability+ gastrointestinal cancers.** A consequence of perfect complementarity between miRNA and mRNA is mRNA cleavage and degradation. Imperfect alignment represses gene translation. ORF: Open reading frame; RISC: RNA-induced silencing complexes.

role in MSI carcinogenesis[45]. Decreased matrix metalloproteinase (MMP)-3 expression due to insertions and/or deletions in the *MMP-3* promoter region led to a decrease in the levels of the active MMP-9 form, which may explain the less invasive potential of MSI+ cancer cells[46] and the propensity for a good prognosis in the case of MSI+ CRCs[47].

Genomic copy number changes are frequently observed in cancers. It is well known that MSI+ cancers show less genomic copy number changes and are mostly diploid[48]. However, genes responsible for chromosomal instability (CIN) could be mutated in MSI+ cancers, and these defects may be selected during the course of cancer progression (Figures 2 and 3)[49]. Furthermore, mutations of *RAD50* and *MRE11* are reportedly associated with defects in nonhomologous end-joining, leading to chromosomal alterations during cancer progression[45].

Altered histone modifications that affect chromatin structures are also involved in carcinogenesis[49]. Epigenetic modifier genes could also be MSI target genes. Ropero *et al*[50] detected frameshift mutations in the histone deacetylase (*HDAC*) *2* gene in MSI+ GI cancers. This *HDAC2* mutation made mutation-positive cancer cells more resistant to the antiproliferative and proapoptotic effects of certain HDAC inhibitors such as trichostatin A, but not to others such as butyric acid and valproic acid. Since HDAC inhibitors may serve as therapeutic agents for cancer, these findings support the use of *HDAC2* mutation status in future pharmacogenetic treatment[50].

## MIRNA PROCESSING MACHINERY GENES AS MSI TARGET GENES

MiRNAs are small noncoding RNAs that regulate gene expression at the posttranscriptional level and are critical in many biological processes and cellular pathways (Figure 4)[11-20]. MiRNA expression profiles of human cancers have been characterized by an overall mature miRNA downregulation[51-53]. The causes of the aberrant miRNA expression patterns in cancer involve DNA copy number amplification or deletion[54], inappropriate transactivation, transcriptional repression by oncogenic and/or other factors[55], failure of miRNA post-transcriptional regulation[56], and genetic mutation[57] or transcriptional silencing associated with hypermethylation of CpG island promoters[58-62].

The control of the miRNA biosynthesis pathway is important in the spatiotemporal pattern of miRNA expression in cells. Thus, impaired miRNA processing pathways

may themselves be targets of genetic and/or epigenetic disruption in cancer[63,64]. Recently, it has been reported that mitogenic signaling can be translated into changes in cell viability and proliferation through the miRNA biogenesis pathway. A *TAR RNA-binding protein 2* (*TARBP2*) gene encodes TRBP, an essential functional partner of DICER1 (Figure 4)[65,66]. TARBP2 is phosphorylated under normal growth conditions, which increases the stability of TARBP2 and DICER[67]. Upon growth factor stimulation, MAPK/ERK pathway increases TARBP2 phosphorylation, leading to a coordinated increase in levels of growth-promoting miRNA and a decrease in the expression of *let-7* tumor suppressor miRNA. In contrast, pharmacological inhibition of MAPK/ERK resulted in an anti-growth miRNA profile[67]. These results further suggest the important role of miRNA processing mediated by TARBP2 in preservation of a normal, untransformed cell state[17].

Melo *et al*[68] have found truncating heterozygous mutations in *TARBP2* in MSI+ cancer cell lines and in primary sporadic and hereditary MSI+ GI cancers (Figure 4). *TARBP2* mutations diminished TRBP protein expression, resulting in impaired miRNA processing and enhanced cellular transformation. The TRBP impairment was associated with a secondary defect in DICER1 activity by destabilization of the DICER1 protein. Thus, *TARBP2* mutations may explain overall miRNA downregulation in a subset of MSI+ cancers. Because the restoration of efficient miRNA production by the reintroduction of TRBP can suppress cancer cell growth, these findings are important for the development of new therapeutic strategies for the treatment of cancer[68].

## MUTATIONS OF THE *EXPORTIN 5* GENE

Because of the nuclear retention of certain precursor miRNAs (pre-miRNAs), mature miRNA expression levels are not consistent with pre-miRNA expression levels in various human cancer cell lines[17]. Thus, defects in the nuclear export of pre-miRNAs may be one of the mechanisms underlying the global impairment of mature miRNAs in human cancer. The *exportin 5* (*XPO5*) mediates nuclear export of pre-miRNA (Figure 4). Melo *et al*[69,70] have identified inactivating heterozygous mutations of *XPO5* in MSI+ cancer cell lines and in primary sporadic and hereditary MSI+ GI cancers. The mutant form of XPO5 does not comprise a C-terminal region that is important for the formation of the pre-miRNA/XPO5/Ran-GTP ternary complex. Thus, the XPO5 defect trapped certain pre-miRNAs in the nucleus, reduced miRNA processing, and impaired miRNA-target inhibition. It is important to note that the restoration of XPO5 functions rescued the disturbed export of critical tumor-suppressive pre-miRNAs, which results in tumor suppression[69].

Interestingly, although the heterozygous *XPO5* mutation decreased accumulation of a fraction of detectable miRNAs, many others were not affected. It seems that XPO5 does not bind to pre-miRNAs universally but has certain substrate preferences, which are possibly mediated by sequence or structure[38]. Strategies directed toward stimulating the activity of miRNA processing factors and restoring the production of mature growth inhibitory miRNAs may have therapeutic value[69].

## MUTATED MIRNA MACHINERY PHENOTYPE AS A NEW CANCER PHENOTYPE

Recent works have suggested that the other component of the miRNA biogenesis pathway, DICER1, is a haploinsufficient tumor suppressor[71,72]. Therefore, it appears that at least three components of the miRNA biogenesis pathway are haploinsufficient tumor suppressors, with TARBP2 and XPO5, but not DICER, mutations prevalent in MSI+ cancers[38]. In addition, the miRISC components *AGO2*, *TNRC6A*, and *TNRC6C* can also be mutated in MSI+ cancers (Figure 4)[73], although the functional significances remain to be determined[38,70].

From these observations, a new cancer phenotype known as mutated miRNA machinery phenotype (MMMP) has been proposed for MSI+ CRCs having mutations in the miRNA machinery genes and the deregulated miRNAome. Although larger studies are required to fully characterize and validate this feature as a criterion for classification, a broader miRNAome-modifying approach may be effective for cancer patients with MMMP[15].

## TRANSCRIPTOMIC DIFFERENCES BETWEEN MSI+ AND MSI- CRCS

As molecular markers, gene expression profiles are being developed for many cancers. Array technology has identified a number of genes that are expressed differentially between MSI+ and MSI- CRCs[74-76]. By using supervised analysis of cDNA microarray data, Giacomini *et al*[77] identified a robust expression signature distinguishing MSI+ and MSI- CRC cell lines. By using high-density oligonucleotide microarrays, Kruhoffer *et al*[78] constructed a gene signature that distinguished MSI+ and MSI- CRCs. The authors further constructed a signature that distinguished sporadic and hereditary cases of MSI+ CRCs. Identification of a signature for MMR deficiency would be relevant, both biologically and clinically[78].

As for miRNAs, Lanza *et al*[79] analyzed 16 MSI+ and 23 MSI- CRCs for genome-wide expression of miRNA and mRNA. On the basis of combined miRNA and mRNA expression, a molecular signature comprising 27 differentially expressed genes, including 8 miRNAs, could correctly distinguish MSI+ and MSI- CRCs. Among the differentially expressed miRNAs, various members of the oncogenic *miR-17-92* family were significantly upregulated in MSI- cancers. Among these, *miR-17-5p*, *miR-20*, *miR-25*, *miR-92-1*, *miR-92-2*, *miR-93-1*, and *miR-106a* were significantly upregulated in MSI- when compared with

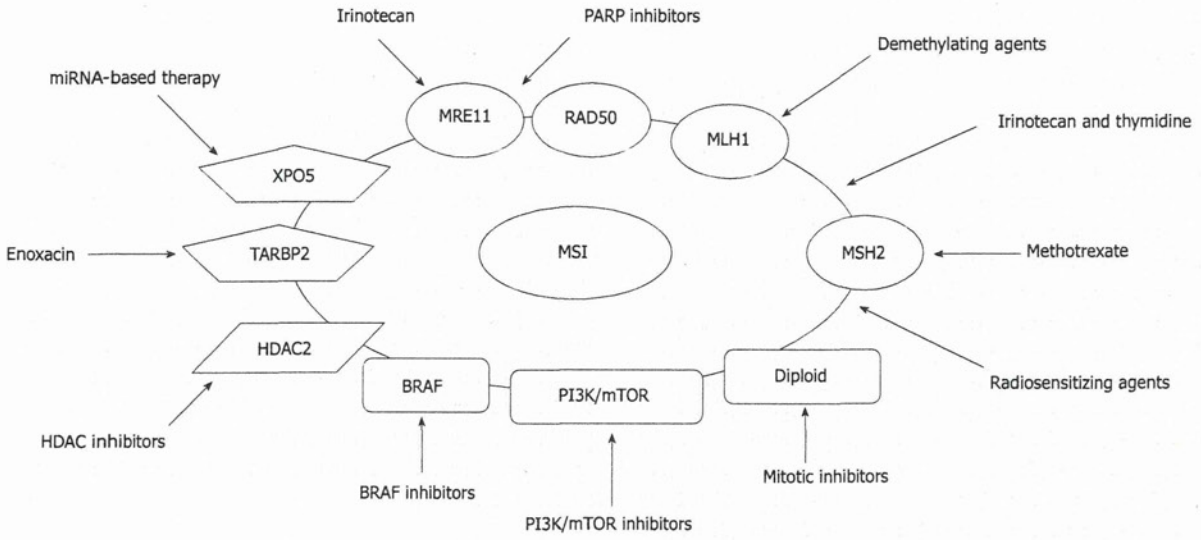


**Figure 5 Targeted therapies based on molecular alterations in microsatellite instability+ colorectal cancers.** Microsatellite instability+ cancers may be managed more effectively with novel targeted therapies based on molecular alterations. MSI: Microsatellite instability; HDAC: Histone deacetylase; PI3K/mTOR: Phosphoinositide 3-kinase/mammalian target of rapamycin; XPO5: Exportin 5.

MSI+ CRCs. Because members of the *miR-17-92* family act as oncogenes, these results may explain, at least in part, the less aggressive behavior of MSI+ CRCs when compared with their MSI- counterparts.

Earle *et al*[80] analyzed 22 MSI-H, including 6 Lynch syndrome, 8 MSI-L, and 25 MSS CRCs for a selected panel of 24 miRNAs. Relative expression of *miR-26b*, *miR-31*, *miR-92*, *miR-155*, *miR-196a*, and *miR-223* were significantly different among MSI subgroups, and *miR-31* and *miR-223* were overexpressed in CRCs of patients with Lynch syndrome. These findings indicate that miRNA expression in CRC is associated with MSI status, including Lynch syndrome and MSI-L, and that miRNAs may play significant roles in these MSI subgroups in addition to having possible effects on cancer characteristics.

Slattery *et al*[81] analyzed 70 CRCs for 866 miRNAs using microarrays. At the 1.5-fold level, 143 miRNAs were differentially expressed in MSI+ CRCs. *MiR-139-3p*, *miR-223*, and *miR-370* were upregulated and *miR-24-2*, *miR-424*, *miR-552*, and *let-7g* were downregulated at a level of 1.5-fold or greater in MSI+ CRCs when compared with MSI- CRCs.

Thus, differentially expressed miRNAs are likely to be relevant, both biologically and clinically, although their functional significances remain to be determined. High levels of *miR-21* in the stroma of CRCs reportedly predict short disease-free survival in stage II CRC patients; however, the levels are not associated with the MSI status[82].

## DEFECTIVE MMR AS A NOVEL THERAPEUTIC TARGET

MSI+ cancers may be managed more effectively with novel targeted therapies based on molecular alterations (Figure 5). A combination of treatments that target both primary alterations of DNA *MMR* gene and secondary alterations, such as frameshift mutations of target genes, may be also effective. A synthetic lethal relationship, where the simultaneous inhibition of two different regulatory pathways leads to cell death, is a recent therapeutic strategy[83]. Therefore, identification of synthetic lethal interactions with MMR deficiency could potentially lead to the identification of specific therapeutic targets[84]. The inhibition of poly (adenosine diphosphate-ribose) polymerase (PARP) is a potential synthetic lethal therapeutic strategy for the treatment of cancers with specific DNA repair defects, such as a BRCA1 or BRCA2 mutation[85].

A subset of MSI+ CRCs may also be suitable for this strategy. A novel PARP inhibitor, ABT-888, showed preferential activity on MSI+ CRC cell lines harboring mutations in both *MRE11* and *RAD50* genes when compared with MSI- cell lines that were wild type for both genes[86]. Recently, Vilar *et al*[87] reported that MSI+ CRCs deficient in double strand break (DSB) repair due to *MRE11* mutations show a higher sensitivity to PARP-1 inhibition. A phase II study assessing the efficacy of a PARP-1 inhibitor, olaparib, in CRCs stratified by MSI status is ongoing. Further clinical studies regarding combinations of a PARP-1 inhibitor with other DSB-inducing therapies, such as radiation or irinotecan, are warranted in MSI+ CRCs with *MRE11* mutations. Although these results need to be confirmed in other settings, they suggest that specific mutations such as *MRE11* can be used to exploit the concept of synthetic lethality in MSI+ cancers, which has been successful in BRCA1-mutant breast cancers[88].

Methotrexate reportedly induces oxidative DNA damage and is selectively lethal to cancer cells with MSH2 defects[84,89]. Thus, a synthetic lethal relationship between deficient MSH2 and treatment with methotrexate led to a phase II trial, incorporating measurement of 8-oxoG

DNA lesions as a biomarker, in metastatic CRC patients with germline mutation or loss of MSH2.

Because MSI+ CRCs often harbor a near diploid stable karyotype, these cancers may be sensitive to mitotic inhibitors, such as taxanes and kinesin-5 inhibitors[90]. To determine the effect of CIN and MSI on the efficacy of the microtubule-stabilizing agent patupilone/EPO960, a phase II study called CIN and Anti-Tubulin Response Assessment in CRC is ongoing. It is assumed that MSI+ CRC patients will benefit more than MSI- CRC patients.

Gene expression signatures can also be used for new MSI+ cancer therapies. Fourteen of the 164 compounds were shown to target MSI+ cancer cell lines using combined gene expression data sets and a connectivity map[91]. Rapamycin, LY-294002, 17-(allylamino)-17-demethoxygeldanamycin, and trichostatin A were the most convincing candidate compounds. MSI+ cell lines with *MLH1* hypermethylation were preferentially targeted by rapamycin and LY-294002 when compared with MSI- cells. These results underscore the relevant role of the PI3K/AKT/mTOR pathway and its therapeutic application in MSI+ cancer, although its clinical significance needs confirmation.

MiRNA-based cancer therapy is limited mainly to targeting a single miRNA[92,93]. However, if most cancers are characterized by a defect in miRNA production and global mature miRNA downregulation[51-53], restoration of the global miRNAome may be an attractive approach in cancer therapy. Melo *et al*[94] have found that the small molecule enoxacin, a fluoroquinolone used as an antibacterial compound, enhances the miRNA-processing machinery by binding to TRBP. Enoxacin was shown to inhibit the growth of a variety of cancer cells. The enhanced miRNA-processing activity by enoxacin did not depend on general fluoroquinolone activity but on the unique chemical structure of enoxacin. These results highlight the key role of disturbed miRNA expression in carcinogenesis, and suggest the potential of novel miRNA-based cancer therapy to restore the disrupted miRNAome of cancer cells.

Finally, it remains to be determined whether *XPO5* mutations can be exploited therapeutically. Since it is difficult to directly restore XPO5 activity, restoring miRNA accumulation by alternative methods may be a more realistic strategy. Given that only a few deregulated tumor suppressor miRNAs appear to be critical for the tumor-promoting effect of *XPO5* mutations, it may be possible to supply those miRNAs exogenously as miRNA duplexes that would not need to undergo nuclear export. It may also be possible to find a subset of important target genes of deregulated tumor suppressor miRNAs, which may be responsive to inactivation by conventional pharmacological methodologies or novel biologics.

## CONCLUSION

The biological and clinical implications of MSI in GI cancers continue to develop. Recent findings, such as overexpression of *miR-21* and *miR-155* and mutations of *TARBP2* and *XPO5* in MSI+ GI cancers, further suggest the important interrelationship between MSI and miRNA in MSI carcinogenesis. The clinicopathological, genetic, epigenetic, prognostic, and therapeutic characteristics of MSI+ cancers are becoming clear, but remain to be fully determined. Analysis of MSI status in cancer patients is warranted as a screening for Lynch syndrome; it could be a potential predictive marker of response to chemotherapy. Since molecular targeting therapeutics are being used in clinical settings and trials, it seems important to clarify if molecular target genes are differentially regulated between MSI+ and MSI- cancers and if the MSI status has the prognostic or predictive significance in metastatic CRC. Further analysis is required to gain insight into MSI carcinogenesis, for a better understanding of disease pathogenesis, and for the development of new diagnostic and/or therapeutic approaches targeting essential pathogenetic alterations.

## REFERENCES


1 **Yamamoto H**, Imai K, Perucho M. Gastrointestinal cancer of the microsatellite mutator phenotype pathway. *J Gastroenterol* 2002; **37**: 153-163
2 **Perucho M**. Tumors with microsatellite instability: many mutations, targets and paradoxes. *Oncogene* 2003; **22**: 2223-2225
3 **Imai K**, Yamamoto H. Carcinogenesis and microsatellite instability: the interrelationship between genetics and epigenetics. *Carcinogenesis* 2008; **29**: 673-680
4 **Sinicrope FA**, Sargent DJ. Clinical implications of microsatellite instability in sporadic colon cancers. *Curr Opin Oncol* 2009; **21**: 369-373
5 **Poulogiannis G**, Frayling IM, Arends MJ. DNA mismatch repair deficiency in sporadic colorectal cancer and Lynch syndrome. *Histopathology* 2010; **56**: 167-179
6 **Goel A**, Boland CR. Recent insights into the pathogenesis of colorectal cancer. *Curr Opin Gastroenterol* 2010; **26**: 47-52
7 **Vilar E**, Gruber SB. Microsatellite instability in colorectal cancer-the stable evidence. *Nat Rev Clin Oncol* 2010; **7**: 153-162
8 **Hewish M**, Lord CJ, Martin SA, Cunningham D, Ashworth A. Mismatch repair deficient colorectal cancer in the era of personalized treatment. *Nat Rev Clin Oncol* 2010; **7**: 197-208
9 **Boland CR**, Goel A. Microsatellite instability in colorectal cancer. *Gastroenterology* 2010; **138**: 2073-2087.e3
10 **Iacopetta B**, Grieu F, Amanuel B. Microsatellite instability in colorectal cancer. *Asia Pac J Clin Oncol* 2010; **6**: 260-269
11 **Saito Y**, Suzuki H, Hibi T. The role of microRNAs in gastrointestinal cancers. *J Gastroenterol* 2009; **44** Suppl 19: 18-22
12 **Davalos V**, Esteller M. MicroRNAs and cancer epigenetics: a macrorevolution. *Curr Opin Oncol* 2010; **22**: 35-45
13 **Krol J**, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet* 2010; **11**: 597-610
14 **Song B**, Ju J. Impact of miRNAs in gastrointestinal cancer diagnosis and prognosis. *Expert Rev Mol Med* 2010; **12**: e33
15 **Davis-Dusenbery BN**, Hata A. MicroRNA in Cancer: The Involvement of Aberrant MicroRNA Biogenesis Regulatory Pathways. *Genes Cancer* 2010; **1**: 1100-1114
16 **Dong Y**, Wu WK, Wu CW, Sung JJ, Yu J, Ng SS. MicroRNA dysregulation in colorectal cancer: a clinical perspective. *Br J Cancer* 2011; **104**: 893-898
17 **Melo SA**, Esteller M. Dysregulation of microRNAs in cancer: playing with fire. *FEBS Lett* 2011; **585**: 2087-2099
18 **Cortez MA**, Bueso-Ramos C, Ferdin J, Lopez-Berestein G, Sood AK, Calin GA. MicroRNAs in body fluids--the mix

of hormones and biomarkers. *Nat Rev Clin Oncol* 2011; **8**: 467-477
19 **van Kouwenhove M**, Kedde M, Agami R. MicroRNA regulation by RNA-binding proteins and its implications for cancer. *Nat Rev Cancer* 2011; **11**: 644-656
20 **Lopez-Serra P**, Esteller M. DNA methylation-associated silencing of tumor-suppressor microRNAs in cancer. *Oncogene* 2012; **31**: 1609-1622
21 **Edwards RA**, Witherspoon M, Wang K, Afrasiabi K, Pham T, Birnbaumer L, Lipkin SM. Epigenetic repression of DNA mismatch repair by inflammation and hypoxia in inflammatory bowel disease-associated colorectal cancer. *Cancer Res* 2009; **69**: 6423-6429
22 **Hampel H**, Frankel WL, Martin E, Arnold M, Khanduja K, Kuebler P, Nakagawa H, Sotamaa K, Prior TW, Westman J, Panescu J, Fix D, Lockman J, Comeras I, de la Chapelle A. Screening for the Lynch syndrome (hereditary nonpolyposis colorectal cancer). *N Engl J Med* 2005; **352**: 1851-1860
23 **Valeri N**, Gasparini P, Fabbri M, Braconi C, Veronese A, Lovat F, Adair B, Vannini I, Fanini F, Bottoni A, Costinean S, Sandhu SK, Nuovo GJ, Alder H, Gafa R, Calore F, Ferracin M, Lanza G, Volinia S, Negrini M, McIlhatton MA, Amadori D, Fishel R, Croce CM. Modulation of mismatch repair and genomic stability by miR-155. *Proc Natl Acad Sci USA* 2010; **107**: 6982-6987
24 **Marsischky GT**, Filosi N, Kane MF, Kolodner R. Redundancy of Saccharomyces cerevisiae MSH3 and MSH6 in MSH2-dependent mismatch repair. *Genes Dev* 1996; **10**: 407-420
25 **Yan H**, Dobbie Z, Gruber SB, Markowitz S, Romans K, Giardiello FM, Kinzler KW, Vogelstein B. Small changes in expression affect predisposition to tumorigenesis. *Nat Genet* 2002; **30**: 25-26
26 **Valle L**, Serena-Acedo T, Liyanarachchi S, Hampel H, Comeras I, Li Z, Zeng Q, Zhang HT, Pennison MJ, Sadim M, Pasche B, Tanner SM, de la Chapelle A. Germline allele-specific expression of TGFBR1 confers an increased risk of colorectal cancer. *Science* 2008; **321**: 1361-1365
27 **Ahluwalia JK**, Hariharan M, Bargaje R, Pillai B, Brahmachari V. Incomplete penetrance and variable expressivity: is there a microRNA connection? *Bioessays* 2009; **31**: 981-992
28 **de la Chapelle A**. Genetic predisposition to human disease: allele-specific expression and low-penetrance regulatory loci. *Oncogene* 2009; **28**: 3345-3348
29 **Volinia S**, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, Prueitt RL, Yanaihara N, Lanza G, Scarpa A, Vecchione A, Negrini M, Harris CC, Croce CM. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci USA* 2006; **103**: 2257-2261
30 **Valeri N**, Gasparini P, Braconi C, Paone A, Lovat F, Fabbri M, Sumani KM, Alder H, Amadori D, Patel T, Nuovo GJ, Fishel R, Croce CM. MicroRNA-21 induces resistance to 5-fluorouracil by down-regulating human DNA MutS homolog 2 (hMSH2). *Proc Natl Acad Sci USA* 2010; **107**: 21098-21103
31 **Rossi L**, Bonmassar E, Faraoni I. Modification of miR gene expression pattern in human colon cancer cells following exposure to 5-fluorouracil in vitro. *Pharmacol Res* 2007; **56**: 248-253
32 **Woerner SM**, Kloor M, Mueller A, Rueschoff J, Friedrichs N, Buettner R, Buzello M, Kienle P, Knaebel HP, Kunstmann E, Pagenstecher C, Schackert HK, Möslein G, Vogelsang H, von Knebel Doeberitz M, Gebert JF. Microsatellite instability of selective target genes in HNPCC-associated colon adenomas. *Oncogene* 2005; **24**: 2525-2535
33 **Markowitz S**, Wang J, Myeroff L, Parsons R, Sun L, Lutterbaugh J, Fan RS, Zborowska E, Kinzler KW, Vogelstein B. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science* 1995; **268**: 1336-1338
34 **Rampino N**, Yamamoto H, Ionov Y, Li Y, Sawai H, Reed JC, Perucho M. Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. *Science* 1997; **275**: 967-969
35 **Yamamoto H**, Sawai H, Perucho M. Frameshift somatic mutations in gastrointestinal cancer of the microsatellite mutator phenotype. *Cancer Res* 1997; **57**: 4420-4426
36 **Grady WM**, Myeroff LL, Swinler SE, Rajput A, Thiagalingam S, Lutterbaugh JD, Neumann A, Brattain MG, Chang J, Kim SJ, Kinzler KW, Vogelstein B, Willson JK, Markowitz S. Mutational inactivation of transforming growth factor beta receptor type II in microsatellite stable colon cancers. *Cancer Res* 1999; **59**: 320-324
37 **Woerner SM**, Benner A, Sutter C, Schiller M, Yuan YP, Keller G, Bork P, Doeberitz MK, Gebert JF. Pathogenesis of DNA repair-deficient cancers: a statistical meta-analysis of putative Real Common Target genes. *Oncogene* 2003; **22**: 2226-2235
38 **Grosshans H**, Büssing I. MicroRNA biogenesis takes another single hit from microsatellite instability. *Cancer Cell* 2010; **18**: 295-297
39 **Woerner SM**, Yuan YP, Benner A, Korff S, von Knebel Doeberitz M, Bork P. SelTarbase, a database of human mononucleotide-microsatellite mutations and their potential impact to tumorigenesis and immunology. *Nucleic Acids Res* 2010; **38**: D682-D689
40 **Yamamoto H**, Gil J, Schwartz S, Perucho M. Frameshift mutations in Fas, Apaf-1, and Bcl-10 in gastro-intestinal cancer of the microsatellite mutator phenotype. *Cell Death Differ* 2000; **7**: 238-239
41 **Chang DK**, Metzgar D, Wills C, Boland CR. Microsatellites in the eukaryotic DNA mismatch repair genes as modulators of evolutionary mutation rate. *Genome Res* 2001; **11**: 1145-1146
42 **Malkhosyan S**, Rampino N, Yamamoto H, Perucho M. Frameshift mutator mutations. *Nature* 1996; **382**: 499-500
43 **Hugo H**, Cures A, Suraweera N, Drabsch Y, Purcell D, Mantamadiotis T, Phillips W, Dobrovic A, Zupi G, Gonda TJ, Iacopetta B, Ramsay RG. Mutations in the MYB intron I regulatory sequence increase transcription in colon cancers. *Genes Chromosomes Cancer* 2006; **45**: 1143-1154
44 **Giannini G**, Rinaldi C, Ristori E, Ambrosini MI, Cerignoli F, Viel A, Bidoli E, Berni S, D'Amati G, Scambia G, Frati L, Screpanti I, Gulino A. Mutations of an intronic repeat induce impaired MRE11 expression in primary human cancer with microsatellite instability. *Oncogene* 2004; **23**: 2640-2647
45 **Koh KH**, Kang HJ, Li LS, Kim NG, You KT, Yang E, Kim H, Kim HJ, Yun CO, Kim KS, Kim H. Impaired nonhomologous end-joining in mismatch repair-deficient colon carcinomas. *Lab Invest* 2005; **85**: 1130-1138
46 **Morán A**, Iniesta P, de Juan C, González-Quevedo R, Sánchez-Pernaute A, Díaz-Rubio E, Ramón y Cajal S, Torres A, Balibrea JL, Benito M. Stromelysin-1 promoter mutations impair gelatinase B activation in high microsatellite instability sporadic colorectal tumors. *Cancer Res* 2002; **62**: 3855-3860
47 **Morán A**, Iniesta P, de Juan C, García-Aranda C, Díaz-López A, Benito M. Impairment of stromelysin-1 transcriptional activity by promoter mutations in high microsatellite instability colorectal tumors. *Cancer Res* 2005; **65**: 3811-3814
48 **Camps J**, Armengol G, del Rey J, Lozano JJ, Vauhkonen H, Prat E, Egozcue J, Sumoy L, Knuutila S, Miró R. Genome-wide differences between microsatellite stable and unstable colorectal tumors. *Carcinogenesis* 2006; **27**: 419-428
49 **Konishi K**, Issa JP. Targeting aberrant chromatin structure in colorectal carcinomas. *Cancer J* 2007; **13**: 49-55
50 **Ropero S**, Fraga MF, Ballestar E, Hamelin R, Yamamoto H, Boix-Chornet M, Caballero R, Alaminos M, Setien F, Paz MF, Herranz M, Palacios J, Arango D, Orntoft TF, Aaltonen LA, Schwartz S, Esteller M. A truncating mutation of HDAC2 in human cancers confers resistance to histone deacetylase inhibition. *Nat Genet* 2006; **38**: 566-569

51 **Lu J**, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. MicroRNA expression profiles classify human cancers. *Nature* 2005; **435**: 834-838

52 **Calin GA**, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006; **6**: 857-866

53 **Gaur A**, Jewell DA, Liang Y, Ridzon D, Moore JH, Chen C, Ambros VR, Israel MA. Characterization of microRNA expression levels and their biological correlates in human cancer cell lines. *Cancer Res* 2007; **67**: 2456-2468

54 **Calin GA**, Croce CM. MicroRNA-cancer connection: the beginning of a new tale. *Cancer Res* 2006; **66**: 7390-7394

55 **Chang TC**, Yu D, Lee YS, Wentzel EA, Arking DE, West KM, Dang CV, Thomas-Tikhonenko A, Mendell JT. Widespread microRNA repression by Myc contributes to tumorigenesis. *Nat Genet* 2008; **40**: 43-50

56 **Thomson JM**, Newman M, Parker JS, Morin-Kensicki EM, Wright T, Hammond SM. Extensive post-transcriptional regulation of microRNAs and its implications for cancer. *Genes Dev* 2006; **20**: 2202-2207

57 **Jazdzewski K**, Murray EL, Franssila K, Jarzab B, Schoenberg DR, de la Chapelle A. Common SNP in pre-miR-146a decreases mature miR expression and predisposes to papillary thyroid carcinoma. *Proc Natl Acad Sci USA* 2008; **105**: 7269-7274

58 **Saito Y**, Liang G, Egger G, Friedman JM, Chuang JC, Coetzee GA, Jones PA. Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. *Cancer Cell* 2006; **9**: 435-443

59 **Lujambio A**, Ropero S, Ballestar E, Fraga MF, Cerrato C, Setién F, Casado S, Suarez-Gauthier A, Sanchez-Cespedes M, Git A, Spiteri I, Das PP, Caldas C, Miska E, Esteller M. Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. *Cancer Res* 2007; **67**: 1424-1429

60 **Lujambio A**, Calin GA, Villanueva A, Ropero S, Sánchez-Céspedes M, Blanco D, Montuenga LM, Rossi S, Nicoloso MS, Faller WJ, Gallagher WM, Eccles SA, Croce CM, Esteller M. A microRNA DNA methylation signature for human cancer metastasis. *Proc Natl Acad Sci USA* 2008; **105**: 13556-13561

61 **Toyota M**, Suzuki H, Sasaki Y, Maruyama R, Imai K, Shinomura Y, Tokino T. Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer. *Cancer Res* 2008; **68**: 4123-4132

62 **Suzuki H**, Yamamoto E, Nojima M, Kai M, Yamano HO, Yoshikawa K, Kimura T, Kudo T, Harada E, Sugai T, Takamaru H, Niinuma T, Maruyama R, Yamamoto H, Tokino T, Imai K, Toyota M, Shinomura Y. Methylation-associated silencing of microRNA-34b/c in gastric cancer and its involvement in an epigenetic field defect. *Carcinogenesis* 2010; **31**: 2066-2073

63 **Kumar MS**, Lu J, Mercer KL, Golub TR, Jacks T. Impaired microRNA processing enhances cellular transformation and tumorigenesis. *Nat Genet* 2007; **39**: 673-677

64 **Viswanathan SR**, Daley GQ. Lin28: A microRNA regulator with a macro role. *Cell* 2010; **140**: 445-449

65 **Chendrimada TP**, Gregory RI, Kumaraswamy E, Norman J, Cooch N, Nishikura K, Shiekhattar R. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* 2005; **436**: 740-744

66 **Haase AD**, Jaskiewicz L, Zhang H, Lainé S, Sack R, Gatignol A, Filipowicz W. TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. *EMBO Rep* 2005; **6**: 961-967

67 **Paroo Z**, Ye X, Chen S, Liu Q. Phosphorylation of the human microRNA-generating complex mediates MAPK/Erk signaling. *Cell* 2009; **139**: 112-122

68 **Melo SA**, Ropero S, Moutinho C, Aaltonen LA, Yamamoto H, Calin GA, Rossi S, Fernandez AF, Carneiro F, Oliveira C, Ferreira B, Liu CG, Villanueva A, Capella G, Schwartz S, Shiekhattar R, Esteller M. A TARBP2 mutation in human cancer impairs microRNA processing and DICER1 function. *Nat Genet* 2009; **41**: 365-370

69 **Melo SA**, Moutinho C, Ropero S, Calin GA, Rossi S, Spizzo R, Fernandez AF, Davalos V, Villanueva A, Montoya G, Yamamoto H, Schwartz S, Esteller M. A genetic defect in exportin-5 traps precursor microRNAs in the nucleus of cancer cells. *Cancer Cell* 2010; **18**: 303-315

70 **Melo SA**, Esteller M. A precursor microRNA in a cancer cell nucleus: get me out of here! *Cell Cycle* 2011; **10**: 922-925

71 **Kumar MS**, Pester RE, Chen CY, Lane K, Chin C, Lu J, Kirsch DG, Golub TR, Jacks T. Dicer1 functions as a haploinsufficient tumor suppressor. *Genes Dev* 2009; **23**: 2700-2704

72 **Hill DA**, Ivanovich J, Priest JR, Gurnett CA, Dehner LP, Desruisseau D, Jarzembowski JA, Wikenheiser-Brokamp KA, Suarez BK, Whelan AJ, Williams G, Bracamontes D, Messinger Y, Goodfellow PJ. DICER1 mutations in familial pleuropulmonary blastoma. *Science* 2009; **325**: 965

73 **Kim MS**, Oh JE, Kim YR, Park SW, Kang MR, Kim SS, Ahn CH, Yoo NJ, Lee SH. Somatic mutations and losses of expression of microRNA regulation-related genes AGO2 and TNRC6A in gastric and colorectal cancers. *J Pathol* 2010; **221**: 139-146

74 **Mori Y**, Yin J, Sato F, Sterian A, Simms LA, Selaru FM, Schulmann K, Xu Y, Olaru A, Wang S, Deacu E, Abraham JM, Young J, Leggett BA, Meltzer SJ. Identification of genes uniquely involved in frequent microsatellite instability colon carcinogenesis by expression profiling combined with epigenetic scanning. *Cancer Res* 2004; **64**: 2434-2438

75 **Banerjea A**, Ahmed S, Hands RE, Huang F, Han X, Shaw PM, Feakins R, Bustin SA, Dorudi S. Colorectal cancers with microsatellite instability display mRNA expression signatures characteristic of increased immunogenicity. *Mol Cancer* 2004; **3**: 21

76 **Kim H**, Nam SW, Rhee H, Shan Li L, Ju Kang H, Hye Koh K, Kyu Kim N, Song J, Tak-Bun Liu E, Kim H. Different gene expression profiles between microsatellite instability-high and microsatellite stable colorectal carcinomas. *Oncogene* 2004; **23**: 6218-6225

77 **Giacomini CP**, Leung SY, Chen X, Yuen ST, Kim YH, Bair E, Pollack JR. A gene expression signature of genetic instability in colon cancer. *Cancer Res* 2005; **65**: 9200-9205

78 **Kruhøffer M**, Jensen JL, Laiho P, Dyrskjøt L, Salovaara R, Arango D, Birkenkamp-Demtroder K, Sørensen FB, Christensen LL, Buhl L, Mecklin JP, Järvinen H, Thykjaer T, Wikman FP, Bech-Knudsen F, Juhola M, Nupponen NN, Laurberg S, Andersen CL, Aaltonen LA, Ørntoft TF. Gene expression signatures for colorectal cancer microsatellite status and HNPCC. *Br J Cancer* 2005; **92**: 2240-2248

79 **Lanza G**, Ferracin M, Gafà R, Veronese A, Spizzo R, Pichiorri F, Liu CG, Calin GA, Croce CM, Negrini M. mRNA/microRNA gene expression profile in microsatellite unstable colorectal cancer. *Mol Cancer* 2007; **6**: 54

80 **Earle JS**, Luthra R, Romans A, Abraham R, Ensor J, Yao H, Hamilton SR. Association of microRNA expression with microsatellite instability status in colorectal adenocarcinoma. *J Mol Diagn* 2010; **12**: 433-440

81 **Slattery ML**, Wolff E, Hoffman MD, Pellatt DF, Milash B, Wolff RK. MicroRNAs and colon and rectal cancer: differential expression by tumor location and subtype. *Genes Chromosomes Cancer* 2011; **50**: 196-206

82 **Nielsen BS**, Jørgensen S, Fog JU, Søkilde R, Christensen IJ, Hansen U, Brünner N, Baker A, Møller S, Nielsen HJ. High levels of microRNA-21 in the stroma of colorectal cancers predict short disease-free survival in stage II colon cancer patients. *Clin Exp Metastasis* 2011; **28**: 27-38

83 **Kaelin WG**. The concept of synthetic lethality in the context of anticancer therapy. *Nat Rev Cancer* 2005; **5**: 689-698

84 **Martin SA**, Lord CJ, Ashworth A. Therapeutic targeting of

the DNA mismatch repair pathway. *Clin Cancer Res* 2010; **16**: 5107-5113

85 **Fong PC**, Boss DS, Yap TA, Tutt A, Wu P, Mergui-Roelvink M, Mortimer P, Swaisland H, Lau A, O'Connor MJ, Ashworth A, Carmichael J, Kaye SB, Schellens JH, de Bono JS. Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *N Engl J Med* 2009; **361**: 123-134

86 **Vilar E,** Chow A, Raskin L, Iniesta MD, Mukherjee B, Gruber SB. Preclinical testing of the PARP inhibitor ABT-888 in microsatellite instable colorectal cancer. *J Clin Oncol* 2009; **27**: 11028 (abstract)

87 **Vilar E**, Bartnik CM, Stenzel SL, Raskin L, Ahn J, Moreno V, Mukherjee B, Iniesta MD, Morgan MA, Rennert G, Gruber SB. MRE11 deficiency increases sensitivity to poly(ADP-ribose) polymerase inhibition in microsatellite unstable colorectal cancers. *Cancer Res* 2011; **71**: 2632-2642

88 **Farmer H**, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, Santarosa M, Dillon KJ, Hickson I, Knights C, Martin NM, Jackson SP, Smith GC, Ashworth A. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* 2005; **434**: 917-921

89 **Martin SA**, McCarthy A, Barber LJ, Burgess DJ, Parry S, Lord CJ, Ashworth A. Methotrexate induces oxidative DNA damage and is selectively lethal to tumour cells with defects in the DNA mismatch repair gene MSH2. *EMBO Mol Med* 2009; **1**: 323-337

90 **Swanton C**, Caldas C. Molecular classification of solid tumours: towards pathway-driven therapeutics. *Br J Cancer* 2009; **100**: 1517-1522

91 **Vilar E**, Mukherjee B, Kuick R, Raskin L, Misek DE, Taylor JM, Giordano TJ, Hanash SM, Fearon ER, Rennert G, Gruber SB. Gene expression patterns in mismatch repair-deficient colorectal cancers highlight the potential therapeutic role of inhibitors of the phosphatidylinositol 3-kinase-AKT-mammalian target of rapamycin pathway. *Clin Cancer Res* 2009; **15**: 2829-2839

92 **Duchaine TF**, Slack FJ. RNA interference and micro RNA-oriented therapy in cancer: rationales, promises, and challenges. *Curr Oncol* 2009; **16**: 61-66

93 **Bader AG**, Brown D, Winkler M. The promise of microRNA replacement therapy. *Cancer Res* 2010; **70**: 7027-7030

94 **Melo S**, Villanueva A, Moutinho C, Davalos V, Spizzo R, Ivan C, Rossi S, Setien F, Casanovas O, Simo-Riudalbas L, Carmona J, Carrere J, Vidal A, Aytes A, Puertas S, Ropero S, Kalluri R, Croce CM, Calin GA, Esteller M. Small molecule enoxacin is a cancer-specific growth inhibitor that acts by enhancing TAR RNA-binding protein 2-mediated microRNA processing. *Proc Natl Acad Sci USA* 2011; **108**: 4394-4399

**S- Editor** Gou SX **L- Editor** A **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6577

*World J Gastroenterol* 2012 December 7; 18(45): 6577-6586
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


ORIGINAL ARTICLE

# Alterations in the human epidermal growth factor receptor 2-phosphatidylinositol 3-kinase-v-Akt pathway in gastric cancer

Yasutaka Sukawa, Hiroyuki Yamamoto, Katsuhiko Nosho, Hiroaki Kunimoto, Hiromu Suzuki, Yasushi Adachi, Mayumi Nakazawa, Takayuki Nobuoka, Mariko Kawayama, Masashi Mikami, Takashi Matsuno, Tadashi Hasegawa, Koichi Hirata, Kohzoh Imai, Yasuhisa Shinomura

Yasutaka Sukawa, Hiroyuki Yamamoto, Katsuhiko Nosho, Hiroaki Kunimoto, Hiromu Suzuki, Yasushi Adachi, Mayumi Nakazawa, Yasuhisa Shinomura, First Department of Internal Medicine, Sapporo Medical University School of Medicine, Sapporo 060-8543, Japan

Hiromu Suzuki, Department of Molecular Biology, Sapporo Medical University School of Medicine, Sapporo 060-8543, Japan

Takayuki Nobuoka, Koichi Hirata, First Department of Surgery, Sapporo Medical University School of Medicine, Sapporo 060-8543, Japan

Mariko Kawayama, Masashi Mikami, Department of Gastroenterology, JR Sapporo Hospital, Sapporo 060-8543, Japan

Takashi Matsuno, Department of Surgery, Sapporo Gekakinen Hospital, Sapporo 060-8543, Japan

Tadashi Hasegawa, Department of Surgical Pathology, Sapporo Medical University Hospital, Sapporo 060-8543, Japan

Kohzoh Imai, Division of Cancer Research, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan

Author contributions: Sukawa Y and Yamamoto H designed the research, performed experiments, analyzed the data and wrote the manuscript; Nosho K, Suzuki H and Adachi Y analyzed the data; Kunimoto H and Nakazawa M performed experiments; Nobuoka T, Kawayama M, Mikami M, Matuno T, Hasegawa T and Hirata K provided the collection of the human material and analyzed the data; and Imai K and Shinomura Y edited the manuscript.

Supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, to Yamamoto H and Shinomura Y

Correspondence to: Hiroyuki Yamamoto, MD, FJSIM, PhD, First Department of Internal Medicine, Sapporo Medical University School of Medicine, S-1 W-16 Chuo-ku, Sapporo 060-8543, Japan. h-yama@sapmed.ac.jp
Telephone: +81-11-6112111 Fax: +81-11-6112282

Received: June 18, 2012 Revised: August 21, 2012
Accepted: August 25, 2012
Published online: December 7, 2012

## Abstract

**AIM:** To investigate human epidermal growth factor receptor 2 (HER2)-phosphatidylinositol 3-kinase (PI3K)-v-Akt murine thymoma viral oncogene homolog signaling pathway.

**METHODS:** We analyzed 231 formalin-fixed, paraffin-embedded gastric cancer tissue specimens from Japanese patients who had undergone surgical treatment. The patients' age, sex, tumor location, depth of invasion, pathological type, lymph node metastasis, and pathological stage were determined by a review of the medical records. Expression of HER2 was analyzed by immunohistochemistry (IHC) using the HercepTest™ kit. Standard criteria for HER2 positivity (0, 1+, 2+, and 3+) were used. Tumors that scored 3+ were considered HER2-positive. Expression of phospho Akt (pAkt) was also analyzed by IHC. Tumors were considered pAkt-positive when the percentage of positive tumor cells was 10% or more. PI3K, catalytic, alpha polypeptide (PIK3CA) mutations in exons 1, 9 and 20 were analyzed by pyrosequencing. Epstein-Barr virus (EBV) infection was analyzed by *in situ* hybridization targeting EBV-encoded small RNA (EBER) with an EBER-RNA probe. Microsatellite instability (MSI) was analyzed by polymerase chain reaction using the mononucleotide markers BAT25 and BAT26.

**RESULTS:** HER2 expression levels of 0, 1+, 2+ and 3+ were found in 167 (72%), 32 (14%), 12 (5%) and 20 (8.7%) samples, respectively. HER2 overexpression (IHC 3+) significantly correlated with intestinal histological type (15/20 *vs* 98 /205, $P = 0.05$). PIK3CA mutations were present in 20 cases (8.7%) and significantly correlated with MSI (10/20 *vs* 9/211, $P < 0.01$).

The mutation frequency was high (21%) in T4 cancers and very low (6%) in T2 cancers. Mutations in exons 1, 9 and 20 were detected in 5 (2%), 9 (4%) and 7 (3%) cases, respectively. Two new types of PIK3CA mutation, R88Q and R108H, were found in exon1. All PIK3CA mutations were heterozygous missense single-base substitutions, the most common being H1047R (6/20, 30%) in exon20. Eighteen cancers (8%) were EBV-positive and this positivity significantly correlated with a diffuse histological type (13/18 *vs* 93/198, $P$ = 0.04). There were 7 cases of lymphoepithelioma-like carcinomas (LELC) and 6 of those cases were EBV-positive (percent/EBV: 6/18, 33%; percent/all LELC: 6/7, 86%). pAkt expression was positive in 119 (53%) cases but showed no correlation with clinicopathological characteristics. pAkt expression was significantly correlated with HER2 overexpression (16/20 *vs* 103/211, $P$ < 0.01) but not with PIK3CA mutations (12/20 *vs* 107/211, $P$ = 0.37) or EBV infection (8/18 *vs* 103/211, $P$ = 0.69). The frequency of pAkt expression was higher in cancers with exon20 mutations (100%) than in those with exon1 (40%) or exon9 (56%) mutations. One case showed both HER2 overexpression and EBV infection and 3 cases showed both PIK3CA mutations and EBV infection. However, no cases showed both PIK3CA mutations and HER2 overexpression. One EBV-positive cancer with PIK3CA mutation (H1047R) was MSI-positive. Three of these 4 cases were positive for pAkt expression. In survival analysis, pAkt expression significantly correlated with a poor prognosis (hazard ratio 1.75; 95%CI: 1.12-2.80, $P$ = 0.02).

**CONCLUSION:** HER2 expression, PIK3CA mutations and EBV infection in gastric cancer were characterized. pAkt expression significantly correlates with HER2 expression and with a poor prognosis.



**Key words:** Human epidermal growth factor receptor 2; Phosphatidylinositol 3-kinase; Catalytic; Alpha polypeptide; Epstein-Barr virus; Akt; Gastric cancer

**Peer reviewer:** Takaaki Arigami, MD, PhD, Department of Surgical Oncology and Digestive Surgery, Field of Oncology, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 891-0175, Japan

Sukawa Y, Yamamoto H, Nosho K, Kunimoto H, Suzuki H, Adachi Y, Nakazawa M, Nobuoka T, Kawayama M, Mikami M, Matsuno T, Hasegawa T, Hirata K, Imai K, Shinomura Y. Alterations in the human epidermal growth factor receptor 2-phosphatidylinositol 3-kinase-v-Akt pathway in gastric cancer. *World J Gastroenterol* 2012; 18(45): 6577-6586 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i45/6577.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6577

## INTRODUCTION

Gastric cancer is one of the most common cancer types and the second leading cause of cancer-related deaths worldwide[1]. Genetic and epigenetic alterations play important roles in the development and progression of these tumors[1,2]. Considerable attention has been given to the potential role of the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway in gastric cancer[3,4]. Various alterations, such as activation of growth factor receptors, PI3K, catalytic, alpha polypeptide (PIK3CA) mutations and inactivation of phosphatase and tensin homolog (PTEN) lead to activation of the PI3K-Akt signaling pathway. With regards to growth factor receptors, there is growing evidence that human epidermal growth factor receptor 2 (HER2) is a key driver of tumorigenesis and an important biomarker in gastric cancer. The amplification or overexpression of HER2 is observed in 7%-34% of these cases[5-9].

PIK3CA is mutated in a wide variety of human tumor types[10,11], including gastric cancers[12-15]. Activating mutations in this gene up-regulate the PI3K-Akt signaling pathway, making it a potentially useful therapeutic target. For example, oncogenic mutations of PIK3CA reportedly render breast cancers more resistant to treatment with the anti-HER2 receptor antibody trastuzumab[16]. Thus, this signaling pathway is thought to be one of the mechanisms underlying resistance to trastuzumab. Trastuzumab has recently been approved for treatment of advanced gastric cancers[5,6].

Pyrosequencing-based methods facilitate the identification of low-frequency tumor mutations and allow a more accurate assessment of tumor mutation burden[17]. PIK3CA mutations have been detected in 4%-25% of gastric cancers[12-15]. However, in most previous studies, only exons 9 and 20 hot spot mutations in PIK3CA were analyzed by DNA sequencing. Moreover, the association between HER2 expression and PIK3CA mutations in gastric cancer has not been reported.

A significant correlation has been found between Epstein-Barr virus (EBV) and the methylation of multiple genes in gastric cancers[18-20]. EBV infection reportedly induces PTEN expression loss through CpG island methylation of its promoter, leading to activation of the PI3K-Akt signaling pathway, in EBV-associated gastric cancer[21].

The aim of our present study was to systematically characterize HER2 expression, PIK3CA mutations, and EBV infection, all of which are involved in the PI3K-Akt signaling pathway, in a large cohort of gastric cancers ($n$ = 231). We wished to determine the prevalence of each of these factors with a high precision and thereby correlate them with clinicopathological and molecular features of gastric lesions, including microsatellite instability (MSI) and phospho Akt (pAkt) expression.

## MATERIALS AND METHODS

### Tissue samples

A total of 231 formalin-fixed, paraffin-embedded (FFPE) gastric cancer tissue specimens from Japanese patients who had undergone surgical treatment was analyzed in

**Table 1 Clinicopathological characteristics of patients with gastric cancer**

| Variables ($n$ = 231) | | $n$ (%) |
|---|---|---|
| Sex | Male | 157 (68) |
| | Female | 74 (32) |
| Age (yr) | Median (range) | 71 (25-91) |
| Location | Cardias | 82 (35) |
| | Body | 62 (27) |
| | Antrum | 83 (36) |
| | Unknown | 4 (2) |
| Depth of invasion | T2 | 125 (54) |
| | T3 | 92 (40) |
| | T4 | 14 (6) |
| Lymph node metastasis | N0 | 65 (28) |
| | N+ | 158 (68) |
| | N1 | 73 (32) |
| | N2 | 56 (24) |
| | N3 | 29 (13) |
| | Unknown | 8 (3) |
| Stage | I B | 49 (21) |
| | II | 45 (19) |
| | IIIA + IIIB | 82 (35) |
| | IV | 51 (22) |
| | Unknown | 4 (2) |
| Lauren histotype | Intestinal | 113 (49) |
| | Diffuse | 112 (48) |
| | Others | 6 (3) |

this study. The patients' age, sex, tumor location, depth of invasion, pathological type, lymph node metastasis, and pathological stage were determined by a review of their medical records. Clinicopathological findings were determined according to the criteria of the Japanese Research Society for Gastric Cancer (Table 1). Our institutional review committee approved the study.

### Immunohistochemistry

HER2 expression was analyzed using the HercepTest™ kit (DAKO, Carpinteria, CA) by manual sample processing in accordance with the manufacturer's instructions. Standard criteria for HER2 positivity (0, 1+, 2+ and 3+) were used. Tumors that scored 3+ were considered HER2-positive. For the immunohistochemical analysis of pAkt, FFPE specimens were processed using SignalStain Boost Detection Reagent (Cell Signaling Technology, Beverly, MA). Briefly, 5-μm-thick sections were dewaxed in xylene, rehydrated in ethanol, and heated with target retrieval solution (DAKO) in an autoclave for antigen retrieval. Endogenous peroxidase was blocked by incubation with 0.3% hydrogen peroxide in methanol for 10 min. The tissue sections were then washed twice with tris-buffered saline (TBS) and preblocked with 10% goat serum in TBS for 60 min. After washing with TBS, the sections were incubated with an anti-phospho-Akt (Ser473) polyclonal antibody (D9E, Cell Signaling Technology) at a dilution of 1:100 for 30 h at 4 ℃. The sections were washed three times in TBS and incubated with SignalStain Boost Detection Reagent for 45 min. After three further washes in TBS, a diamino-benzidine tetrahydrochloride working solution was applied. Finally, the sections were counterstained with hematoxylin. Tumors were considered pAkt-positive when the percentage of positive tumor cells was 10% or more[22]. Only clear staining of the tumor cell nucleus and/or cytoplasm was considered positive.

### Mutation analysis of the PIK3CA gene by pyrosequencing

Genomic DNA was extracted from tumor specimens and mutations in exon9 and exon20 of the *PIK3CA* gene were analyzed by pyrosequencing as described previously[23,24]. We also developed a pyrosequencing assay to detect PIK-3CA exon1 mutations using the primer sets exon1-RS1 (5'-GGGAAGAATTTTTTGATGAAACA-3' for the biotinylated forward primer and 5'-GGTTGCCTACT-GGTTCAATTACTT-3' for the reverse primer) and exon1-RS2 (5'-CGGCTTTTTCAACCCTTTTT-3' for the forward primer and 5'-ATTTCTCGATTGAG-GATCTTTTCT-3' for the biotinylated reverse primer). Each polymerase chain reaction (PCR) mix contained the forward and reverse primers (each 10 μmol/L), a 25 mmol/L dNTP mix with dUTP, 75 mmol/L $MgCl_2$, 1 × PCR buffer, 1.0 U of exTaq, and 2 μL of template DNA in a total volume of 25 μL. PCR conditions were as follows: initial denaturing at 95 ℃ for 5 min; 50 cycles of 94 ℃ for 20 s, 50 ℃ for 20 s and 74 ℃ for 40 s; and a final extension at 72 ℃ for 1 min. The PCR products (each 25 μL) were sequenced using the PyroMark kit and Pyrosequencing PSQ96 HS System (Qiagen, Valencia, CA).

### In situ hybridization for EBER1

The presence of EBV in the carcinoma tissues was evaluated by *in situ* hybridization (ISH) targeting of EBV-encoded small RNA (EBER-ISH) with an EBER-RNA probe (Dako Cytomation).

### Microsatellite instability analysis

MSI was analyzed by PCR using the mononucleotide markers (BAT25 and BAT26). Based on the number of markers showing instability per tumor sample, cancers were divided into two groups; those with one or more of the two markers displaying MSI and those with no instability (microsatellite stable).

### Statistical analysis

For all statistical analysis, the JMP program was used. All $P$ values were two-sided and statistical significance was set at $P \leq 0.05$. For categorical data, the $\chi^2$ test was used. For survival analysis, Kaplan-Meier method and log-rank test were used. For analysis of cancer-specific mortality, we excluded surgery-related deaths (deaths within one month of surgery).

## RESULTS

### HER2 expression in gastric cancer tissues

HER2 expression levels of 0, 1+, 2+ and 3+ were found in 167 (72%), 32 (14%), 12 (5%) and 20 (8.7%) samples, respectively (Figure 1). HER2 overexpression (IHC 3+) significantly correlated with intestinal histological type

**Table 2 Clinicopathological characteristics of patients with gastric cancer based on human epidermal growth factor receptor 2 expression, phosphatidylinositol 3-kinase, catalytic, alpha polypeptide mutations and Epstein-Barr virus infection *n* (%)**

| | | HER2 | | | PIK3CA | | | EBV | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Positive (*n* = 20) | Negative (*n* = 211) | *P* value | Mutation (*n* = 20) | Wild type (*n* = 211) | *P* value | Positive (*n* = 18) | Negative (*n* = 204) | *P* value |
| Sex | Male | 15 (75) | 142 (67) | 0.48 | 13 (65) | 144 (68) | 0.77 | 14 (78) | 138 (68) | 0.38 |
| | Female | 5 (25) | 69 (33) | | 7 (35) | 70 (32) | | 4 (22) | 66 (32) | |
| Age | Median | 69 (50-84) | 71 (25-91) | 0.26 | 71 (25-85) | 70 (38-91) | 0.40 | 72 (48-90) | 70 (38-91) | 0.41 |
| Location | Cardias | 10 (50) | 72 (34) | 0.49 | 5 (25) | 77 (36) | 0.31 | 8 (44) | 73 (36) | 0.70 |
| | Body | 5 (25) | 57 (27) | | 4 (20) | 58 (27) | | 5 (28) | 55 (27) | |
| | Antrum | 5 (25) | 78 (37) | | 10 (50) | 73 (35) | | 5 (28) | 75 (37) | |
| | Unknown | 0 | 4 (2) | | 1 (5) | 2 (1) | | 0 | 1 (0) | |
| Depth | T2 | 12 (60) | 113 (54) | 0.48 | 8 (40) | 117 (55) | 0.15 | 12 (67) | 106 (52) | 0.35 |
| | T3 | 8 (40) | 84 (40) | | 9 (45) | 83 (39) | | 6 (33) | 85 (42) | |
| | T4 | 0 | 14 (6) | | 3 (15) | 11 (5) | | 0 | 13 (6) | |
| L/N meta | N0 | 5 (25) | 60 (28) | 0.71 | 4 (20) | 61 (29) | 0.37 | 3 (17) | 57 (28) | 0.28 |
| | N+ | 14 (70) | 144 (68) | | 16 (80) | 142 (67) | | 14 (77) | 140 (69) | |
| | N1 | 5 (25) | 68 (32) | | 8 (40) | 65 (31) | | 8 (44) | 63 (31) | |
| | N2 | 6 (30) | 50 (24) | | 6 (30) | 50 (24) | | 2 (11) | 53 (26) | |
| | N3 | 3 (15) | 26 (12) | | 2 (10) | 27 (13) | | 4 (22) | 24 (12) | |
| | Unknown | 1 (5) | 7 (3) | | 0 | 8 (4) | | 1 (6) | 7 (3) | |
| Stage | I | 5 (25) | 44 (21) | 0.89 | 1 (5) | 48 (23) | 0.14 | 3 (17) | 41 (20) | 0.98 |
| | II | 3 (15) | 42 (20) | | 7 (35) | 38 (18) | | 4 (22) | 39 (19) | |
| | III | 6 (30) | 76 (36) | | 8 (40) | 74 (35) | | 6 (33) | 75 (37) | |
| | IV | 5 (25) | 46 (22) | | 4 (20) | 47 (22) | | 4 (22) | 46 (23) | |
| | Unknown | 1 (5) | 3 (1) | | 0 | 4 (2) | | 1 (6) | 3 (1) | |
| Lauren | Intestinal | 15 (75) | 98 (46) | 0.05 | 14 (70) | 99 (47) | 0.13 | 5 (28) | 105 (51) | 0.04 |
| histotype | Diffuse | 5 (25) | 107 (51) | | 6 (30) | 106 (50) | | 13 (72) | 93 (46) | |
| | LELC | 0 | 6 (3) | | 2 (10) | 4 (2) | | 5 (28) | 0 | |
| | Others | 0 | 6 (3) | | 0 | 6 (3) | | 0 | 6 (3) | |
| MSI | | 2 (10) | 28 (13) | 0.72 | 10 (50) | 20 (9) | < 0.01 | 1 (6) | 26 (13) | 0.36 |
| pAkt | | 16 (84) | 103 (51) | < 0.01 | 12 (63) | 107 (53) | 0.37 | 8 (47) | 103 (52) | 0.69 |
| 3 yr OS (%) | | 29.4 | 59.2 | 0.24 | 57.3 | 56.8 | 0.59 | 57.4 | 57.3 | 0.98 |

MSI: Microsatellite instability; LELC: Lymphoepithelioma-like carcinoma; HER2: Human epidermal growth factor receptor 2; PIK3CA: Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide mutations; EBV: Epstein-Barr virus; pAkt: Phospho Akt; OS: Overall survival.

**Table 3 Frequencies of phosphaddylinositol 3-kinase, catalytic, alpha polypeptide mutations detected in gastric cancer tissues**

| | Mutation | Overall frequency | Percent/ total cases | Percent/ mutated cases | Microsatellite instability |
|---|---|---|---|---|---|
| Exon1 | R88Q | 1 | 0.4 | 5 | 1 |
| | R108H | 4 | 1.7 | 20 | 1 |
| | Total | 5 | 2.2 | | 2 |
| Exon9 | E542K | 5 | 2.2 | 25 | 1 |
| | E545K | 2 | 0.9 | 10 | 1 |
| | E545G | 2 | 0.9 | 10 | 1 |
| | Total | 9 | 4.0 | | 3 |
| Exon20 | H1047Y | 1 | 0.4 | 5 | 1 |
| | H1047R | 6 | 2.6 | 30 | 4 |
| | Total | 7 | 3.0 | | 5 |

(15/20 *vs* 98/205, $P = 0.05$, Table 2). Three-year survival rates were 29% in patients with HER2 overexpression and 59% in cases without HER2 overexpression, respectively [hazard ratio (HR) 1.73; 95%CI: 0.87-3.14, $P = 0.24$].

### Mutations of the PIK3CA gene in gastric cancer tissues

PIK3CA mutations were present in 20 cases (8.7%) (Table 2 and Figure 2). The mutation frequency was high (21%) in T4 cancers and low (6%) in T2 cancers. Mutations in exons 1, 9 and 20 of PIK3CA were detected in 5 (2%), 9 (4%) and 7 (3%) cases, respectively (Table 3). One case had multiple PIK3CA mutations (R108H and E542K). The exon20/exon9 prevalence ratio was 0.78 (7/9). Two new types of PIK3CA mutations, R88Q and R108H, were detected in exon1. All mutations were heterozygous missense single-base substitutions and the most common mutation was H1047R (6/20; 30%) in exon20. PIK3CA mutations were also found to significantly correlate with MSI (10/20 *vs* 9/211, $P < 0.01$) but not with other clinicopathological characteristics. The three-year survival rates were 57% in patients with PIK3CA mutations and 57% in cases without PIK3CA mutations, respectively (HR 1.37; 95%CI: 0.68-3.26, $P = 0.59$).

### EBV infection

Eighteen samples in our cohort (8%) were EBV-positive and this positivity significantly correlated with diffuse histological type (13/18 *vs* 93/198, $P = 0.04$) (Table 2 and Figure 3). There were 7 cases of LELC and 6 of those cases were EBV-positive (percent/EBV: 6/18, 33%; percent/all LELC: 6/7, 86%). The three-year survival rates were 57% in patients with EBV infection and 57% in those without EBV infection (HR 0.81; 95%CI: