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Research in Veterinary Science 74 (2003) 101–104

RVS

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Short communication

Genomic hypomethylation in neoplastic cells from dogs with malignant lymphoproliferative disorders

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Accepted 1 November 2002

Abstract

DNA methylation is an epigenetic modification in which a methyl group is added usually to the fifth carbon position of a cytosine residue. Dysregulation of this process is an important molecular event which has been shown to be associated with neoplastic transformation and tumour progression in humans and mice. Features of methylation dysregulation in many different types of neoplasms include general genomic hypomethylation, focal hypermethylation, and altered expression of genes which encode a series of DNA (cytosine-5) methyltransferases. Interestingly, many types of neoplasia that are recognised in humans also develop spontaneously in the dog. By comparing the restriction patterns of *Msp*I and *Hpa*II, this study demonstrates that as in human, genomic hypomethylation is a feature of neoplastic cells in the majority of canine lymphoma cases and approximately one-third of canine leukemia cases confirming that dysregulation of the DNA methylating machinery is implicated in malignant transformation of lymphoid cells in some dogs.

1. Introduction

Cytosine methylation is an essential (Li et al., 1992) epigenetic DNA modification which controls many cellular functions including gene transcription (Holliday and Ho, 1998). Gene activity is generally associated with cytosine hypomethylation, whereas cytosine methylation contributes towards gene silencing. DNA methylation is also involved in genomic stabilisation (Jeppesen and Turner, 1993), chromatin organisation (Keshet et al., 1986), genomic imprinting (Surani, 1998) and possibly silencing of integrated foreign DNA (Yoder et al., 1997). The diverse range of critical cellular functions in which DNA methylation is involved highlights the biological significance of this process.

As cytosine methylation has the ability to influence gene expression (Holliday and Ho, 1998), dysregulation of this process is likely to effect regulation of the cell cycle. It is therefore not surprising that methylation dysregulation has been shown to be associated with neoplastic transformation and tumorigenesis in both humans and mice (Kay et al., 1997; Laird et al., 1995).

Extensive genomic hypomethylation is one of the well recognised features of neoplastic cells in many different types of tumours. It has been suggested that as a result of genomic hypomethylation, some proto-oncogenes may lose quiescence and thus induce tumorigenesis (see Kay et al., 1997).

Many forms of neoplasia, including malignant lymphoproliferative disease, develop spontaneously in both humans and dogs (Teske, 1994). As our previous work, and that of others, demonstrated that methylation dysregulation is a characteristic feature of neoplastic lymphoid cells in humans (Baylin et al., 1987; Kay et al., 1995; Taylor et al., 2001), methylation dysregulation may also play a role in the development of malignant lymphoproliferative disorders in the dog. In order to explore this possibility, the present study was undertaken to determine whether general genomic hypomethylation is a feature of neoplastic cells in canine lymphoma and leukemia.

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PII: S0034-5288(02)00179-0

In mammalian cells, methylated cytosine residues predominantly occur within the context of a 5′-CpG-3′ dinucleotide. In contrast to its isoschizomer *Msp*I, hydrolysis by the methylation sensitive restriction endonuclease, *Hpa*II, is inhibited by methylation of the cytosine residue within the 5′-CpG-3′ dinucleotides in their recognition sequence, 5′-CCGG-3′. Thus, fewer methylated cytosine residues within 5′-CpG-3′ sites in genomic DNA would result in generation of a smaller sized range of DNA restriction fragments following *Hpa*II digestion.

Samples of genomic DNA isolated using a standard phenol/chloroform extraction and ethanol precipitation protocol (Maniatis et al., 1982) from the peripheral blood leukocytes (PBL) and affected lymph nodes of dogs with lymphoma as well as the PBL of healthy adult dogs and dogs with leukemia were therefore digested with *Hpa*II and electrophoresed on a 1.8% agarose gel for approximately 200 V/h. Comparison of the *Hpa*II restriction fragment patterns was used to determine alterations in the methylation status of the genome.

Methylation score values (MSVs) were assigned according to the method described by Wahlfors and colleagues (1992) in which following electrophoresis the proportion of fragments within 2.0–2.3-kb were compared between *Msp*I and *Hpa*II digests of genomic DNA. Background values were determined by measuring the intensity of fragments in the 2.0–2.3-kb region of DNA samples which were incubated without enzyme. Intensities were determined using a Computing Densitometer with a Molecular Dynamics software program. The equation used to determine MSVs is as follows:

$$\mathrm{MSV} = \frac{\mathrm{Density}_{\mathit{Hpa}\mathrm{II}} - \mathrm{Density}_{\mathrm{Background}}}{\mathrm{Density}_{\mathit{Msp}\mathrm{I}} - \mathrm{Density}_{\mathrm{Background}}}.$$

Statistical significance between MSVs was determined using a two-sample *t* tests where *t* has a Student's *t*-distribution (Table 1).

Comparison of the patterns of restriction fragments observed following digestion of genomic DNA isolated from the PBL of healthy adult dogs with *Msp*I and the methylation sensitive restriction endonuclease, *Hpa*II (Fig. 1a) indicates that in the canine genome many 5′-CpG-3′ sites within 5′-CCGG-3′ recognition sequences are methylated. 5′-CpG-3′ methylation has been previously described in many other mammalian species, including humans (Bird, 1980).

Table 1
Methylation score values between the sample groups

Sample group	Mean MSV[a]	No. of cases	p[b]
Healthy PBL	0.465	10	–
Lymphoma PBL	0.529	12	>0.25
Leukemia PBL	0.697	8	<0.05
Lymphoma lymph node	0.584	10	<0.01

[a] Methylation score value.
[b] *p*-Value obtained when the mean methylation score value of a sample group is compared to the mean methylation score value of the healthy PBL sample group using a two-sample *t* test.

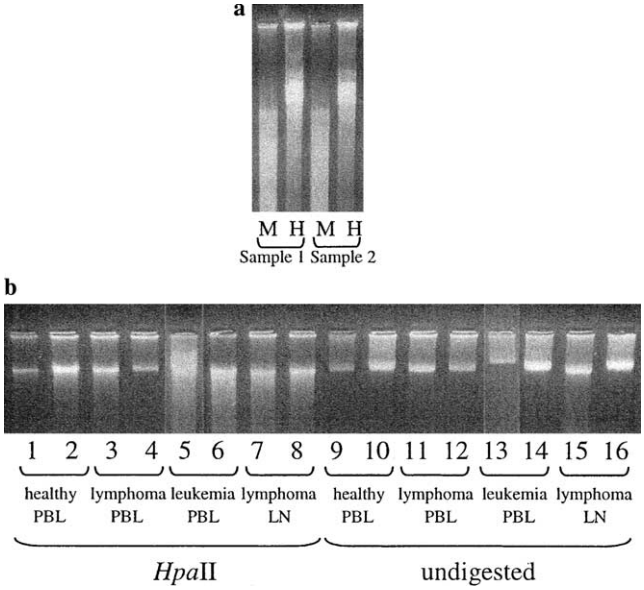

Fig. 1. (a) Demonstration of the *Msp*I (M) and *Hpa*II (H) restriction fragment patterns following digestion of genomic DNA isolated from the PBL of healthy adult dogs. Differences between the patterns of restriction fragments following *Msp*I digestion compared to hydrolysis by *Hpa*II indicates that many CpG sites within the recognition sequence 5′-CCGG-3′ are normally methylated in the adult canine genome. (b) Examples of *Hpa*II restriction fragment patterns following digestion of genomic DNA isolated from the PBL of healthy adult dogs (lanes 1–2), PBL of dogs with leukemia associated with genomic hypomethylation (lanes 5–6) and the PBL (lanes 3–4) and affected lymph nodes (lanes 7–8) of dogs with lymphoma associated with genomic hypomethylation. In order to exclude the possibility that the *Hpa*II restriction fragment patterns observed in lanes 1–8 may be the result of DNA degradation, the integrity of undigested DNA of the same samples was compared (lanes 9–16).

More importantly, the results of this study also demonstrate that the methylation status of the canine genome, as determined by *Hpa*II digestion, is unaltered in non-neoplastic cells isolated from the PBL of dogs with lymphoma (Fig. 1b, lanes 3 and 4). These conclusions are supported by reference to MSVs found in PBL of healthy adult dogs and PBL isolated from dogs with lymphoma (see Table 1).

Comparison between the *Hpa*II restriction fragment patterns generated following digestion of genomic DNA isolated from the PBL of dogs with leukemia (Fig. 1b, lanes 5 and 6) and neoplastic cells isolated from the affected lymph nodes of dogs with lymphoma (Fig. 1b, lanes 7 and 8) with appropriate controls (Fig. 1b, lanes 1–4, respectively) indicates that more *Hpa*II recognition sequences in genomic DNA from the malignant lymphoid cells are able to be hydrolysed with *Hpa*II compared to non-neoplastic lymphoid cells.

In order to exclude the possibility that differences in the *Hpa*II restriction fragment patterns observed between neoplastic and normal cells (Fig. 1b, lanes 1–8) were the result of variation in the integrity of genomic DNA between samples, the electrophoretic mobility of undigested DNA samples was examined. As shown in lanes 9–16 (Fig. 1b), respectively, the integrity of undigested DNA was similar between each of the samples. These findings permitted the conclusion to be drawn that variation in *Hpa*II digestion profiles between samples was due to differences in the number of hydrolysable *Hpa*II recognition sequences and consequently the number of methylated cytosine residues within 5′-CCGG-3′ recognition sequences.

The significance of the extent of genomic hypomethylation in neoplastic lymphoid cells was determined by reference to the MSVs as previously determined (Wahlfors et al., 1992). As shown in Table 1, MSVs of genomic DNA isolated from PBL of dogs suffering from leukemia and the affected lymph nodes from dogs suffering from lymphoma were significantly different from MSVs in genomic DNA from the PBL of healthy dogs ($p < 0.05$ and < 0.01, respectively).

Analysis of the *Hpa*II pattern of restriction fragments demonstrated genomic hypomethylation of neoplastic cells in 3 out of 10 (30%) cases of canine leukemia and 9 out of 13 (69%) cases of canine lymphoma. Results of these studies have therefore confirmed that, as in humans, methylation dysregulation reflected by genomic hypomethylation is associated with neoplastic transformation and/or disease progression in some canine malignant lymphoproliferative disorders. As might be expected, however, the results of this study support the likelihood that there are multiple underlying molecular genetic pathways resulting in the same disease phenotype in the dog, only some of which involve dysregulation of the DNA methylation machinery.

The inability to demonstrate genomic hypomethylation in neoplastic cells in two-thirds of the cases of leukemia and approximately one-third of the cases of canine lymphoma is also consistent with the view that methylation dysregulation is most likely implicated in the early phases of neoplastic transformation and/or tumour progression rather than reflection of a molecular genetic event consequential to neoplastic transformation (Bagwe et al., 1997) perhaps by permitting inappropriate expression of genes which promote the cell cycle.

These findings have important diagnostic implications. Although methods reported here may be cumbersome for routine diagnostic purposes, based on these findings future studies will identify specific genes which are demethylated exclusively in cells which are malignantly transformed. Moreover, in keeping with the generalised genomic hypomethylation and focal hypermethylation associated with malignant transformation in lymphoid cells in humans, it is predicted that focal genomic hypermethylation, including genes such as *Myf-3* (Taylor et al., 2001) or *calcitonin* (Thomas et al., 1999) will be a molecular genetic feature of malignantly transformed lymphoid cells in the dog. The introduction of more rapid and simpler ways of measuring hypermethylation or hypomethylation of specific regions of the genome will enable routine diagnostic tests to be developed which will not only distinguish between malignant and benign proliferation of canine lymphoid cells but also identify malignant lymphoid disorders in the dog which will benefit from new therapeutic treatment aimed at correcting defects in the underlying molecular genetic mechanisms in dysregulation of the DNA methylating machinery.

Identification of cases of spontaneous malignant lymphoproliferative disease in the dog associated with dysregulation of the DNA methylating machinery will provide an appropriate vehicle for assessment of novel focus of treatment aimed at correction of defective elements of the DNA methylating or demethylating machinery.

Acknowledgments

The authors are grateful to Dr. K. Wyatt for his expertise in obtaining relevant biopsy material from dogs diagnosed with lymphoma, Dr. S. Beetson for collection of the canine leukemia samples, Dr. D. Hargest for supply of the control samples used in this study as well as critical review of the manuscript by A/Prof. T. Gotjamanos. This project was partially funded by a grant from the Australian Companion Animal Health Foundation.

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