The Expression of Low Molecular Weight Protein Tyrosine Phosphatase is Up-regulated in 1,2-Dimethylhydrazine-Induced Colon

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SHORT REPORT

The expression of low molecular weight protein tyrosine phosphatase is up-regulated in 1,2-dimethylhydrazine-induced colon tumours in rats

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Recent studies have assessed the role of low molecular weight protein tyrosine phosphatase (LMW-PTP) in cell transformation and tumour onset and progression, observing a significant increase in the expression of LMW-PTP mRNA and protein in human breast, colon, bladder and kidney tumour samples. Moreover, its enhanced expression is generally prognostic of a more aggressive cancer. To better understand the role of this protein during colon carcinogenesis and to study whether its overexpression is also observed in earlier phases of carcinogenesis, we studied its expression in colon tumours, induced in rats by treatment with 1,2-dimethylhydrazine (DMH), an animal model that resemble the sequential formation of histopathological lesions of spontaneous carcinogenesis in humans. The results show a significant increase in LMW-PTP expression in adenocarcinomas, suggesting that this phenomenon is associated with the onset of malignancy. Moreover a significant overexpression of LMW-PTP transcript is associated with tumours originating in the proximal (right) part of the colon, confirming an observation already reported for human colon cancer.

Key words: low molecular weight protein tyrosine phosphatase; 1,2-dimethylhydrazine; adenocarcinomas; adenomas; colon cancer

Protein tyrosine phosphorylation plays a key role in the generation of signals necessary for different cellular events such as growth, migration and invasion of normal and malignant cells.[1] Tumour onset and progression is clearly correlated with the action of different tyrosine kinases,[1,2] which are also considered reliable markers of cancer progression.[3,4] Recent evidence indicates that phosphotyrosine protein phosphatases (PTPs) are also very important in modulating the cell phosphorylation. Almost 70 different enzymes are recognised to be part of the PTP superfamily,[5] among which low molecular weight protein tyrosine phosphatase (LMW-PTP) is a 18-kDa enzyme that is widely expressed in different tissues (for a review see Raugei *et al.*).[6] LMW-PTP have been shown to interact with several receptor tyrosine kinases and docking proteins, including platelet-derived growth factor receptor (PDGF-R),[7] ephrin A2 receptor (EphA2)[8,9] and β-catenin.[10] The role of LMW-PTP in cell transformation has been investigated by Kikawa *et al.*,[8] who demonstrated that LMW-PTP is frequently overexpressed in many oncogene-transformed or human tumour-derived epithelial cells and that the overexpression of LMW-PTP is sufficient to confer transformation to non-transformed epithelial cells.[8] Chiarugi *et al.*[11] demonstrated that LMW-PTPs act as positive regulators of tumour onset and progression, showing that LMW-PTP-transfected NIH3T3 fibroblasts engrafted in nude mice induce the onset of larger fibrosarcomas, probably due to the LMW-PTP dependent ephrin A2 receptor (EphA2) tyrosine dephosphorylation.[11] This condition is highly associated with many human cancers and has also been observed also in prostate-derived tumour cell lines.[9] Recently, a significant increase in the expression of LMW-PTP mRNA and protein level was observed in malignant samples of breast, colon, bladder and kidney.[12] It was also found that colon cancers with increased LMW-PTP mRNA expression were correlated with unfavorable outcome, thus suggesting that LMW-PTP may be considered an oncogene.

To better understand the role of this protein during colon carcinogenesis and to determine whether over-expression of this gene is also observed in earlier phases of carcinogenesis, such as in adenomas (*i.e.* benign tumours), we studied LMW-PTP expression in a well defined experimental model such as colon tumourigenesis induced in rats by administration of 1,2-dimethylhydrazine (DMH), a specific colon carcinogen. In this model, colon cancer develops through the sequential formation of histopathological lesions similar to those observed in spontaneous carcinogenesis in humans,[13] thus allowing the study of the various stages of cancer formation (preneoplastic lesions, adenomas, carcinomas).

Material and methods

Tumour induction

Male F344 rats were obtained from Nossan, Correzzana, Milan, Italy, housed in plastic cages with wire tops, maintained at a temperature of 22°C, with a 12-hr light-dark cycle. Animal care followed the European Union Regulations on the Care and Use of Laboratory Animals. The experimental protocol was approved by a local Ethical Committee for Animal Experimentation, Florence, Italy and by the Commission for Animal Experimentation of the Ministry of Health, Rome, Italy. To induced carcinogenesis, rats received 10 injections (s.c.) of DMH (30 mg/kg body weight; dissolved in sterile saline and buffered with sodium hydroxide at neutral pH) at 1 week intervals (total dose 300 mg/kg body weight). Animals were sacrificed 16 weeks after the last injection with the carcinogen.

Evaluation of the tumours

At sacrifice, the colon was macroscopically examined for the presence and location of tumours or other pathological lesions. Tissues showing a deviation from normal morphology were fixed in 10% buffered formalin and embedded in paraffin blocks. Samples of tumour tissue and of apparently normal mucosa were also harvested and stored at −80°C until analysis for RT-PCR (see below). Paraffin blocks were sectioned and stained with hematoxylin-eosin to confirm the presence and type of tumours by histopathological examination, which was performed by a pathologist unaware of the codes of the specimens. Before being fixed in formalin, suspected macroscopic lesions were measured with a caliper and their dimensions were calculated by multiplying the two main diameters of each lesion. Cancer histological types were evaluated on the basis of the histotype, grading and pattern of growth; adenomas were classified according to Morson *et al.*[14]

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TABLE I – PRIMERS FOR AMPLIFICATION OF RAT LMW-PTP

Amplicons	Primers
Total	Forward: 5′-GGG TCC AAG TCA GTG CTG TTC-3′
LMW-PTP	Reverse: 5′-CGT TTT CAT CAG TTA CCA ATT TTC TG-3′
	probe(MGB®, FAM™): 5′-TGT GTC TCG GTA ACA TTT GCC GGT CA-3′

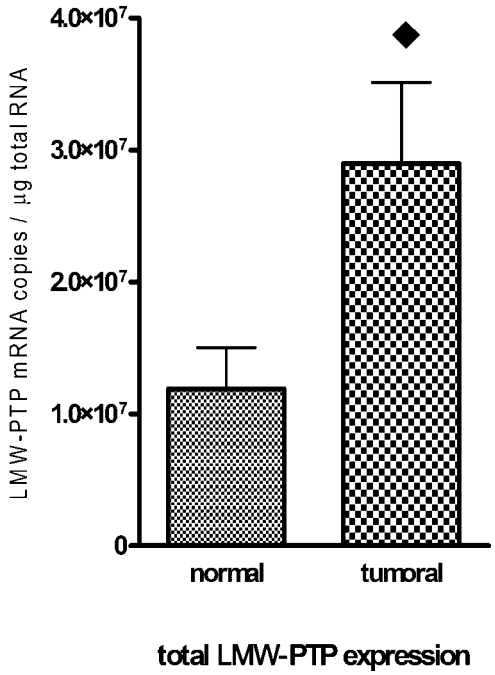

FIGURE 1 – LMW-PTP expression in cancer tissues and in paired normal mucosa. Total LMW-PTP mRNA was subjected to real-time PCR quantitative analysis. $n = 22$. ♦ : significantly different when compared with normal paired mucosa, $p = 0.0016$.

RNA extraction

Tissues were disrupted by homogenization in guanidine isothiocyanate-containing lysis Qiagen buffer (Qiagen, Valencia, CA). Total RNA was extracted with QIAshredder and RNeasy MiniKit Qiagen® columns. RNA was eluted with 50 µl of ribonuclease free water. Samples were treated with ribonuclease free DNase Set® (Qiagen, Valencia, CA) to eliminate DNA. Total RNA concentrations were determined with the GeneQuant spectrophotometer (Amersham Biosciences, GE Healthcare, NJ).

Quantitative mRNA evaluation

The primers-probe set for mRNA quantification with the ABI Prism 7700 Sequence Detection System was selected by the proprietary software Primer Express. To evaluate the total amount of LMW-PTP mRNA, we chose the forward primer at 10 nucleotides from the ATG and the reverse primer starting from nucleotide 82 into the rat coding sequence, while the internal MGB® (TaqMan® Minor Groove Binder; PE Applied Biosystems) probe labelled with FAM™ hybridised on 35–61 nts of coding sequence (Table I). The mRNA of 483 bp (158 amino acids) did not present any intron, and for that reason the FAM probe did not hybridise on the intron–exon junction, as usual. The gene data are available at NCBI Blast Rat sequence website (www.ncbi.nlm.nih.gov/genome/seq/BlastGen/ accession number gi:62650885). The gene is located on chromosome 6 (6q24).

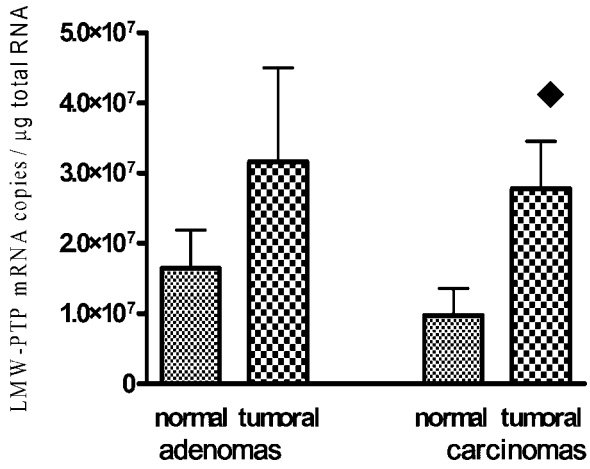

FIGURE 2 – The 22 total samples were sorted according to their histopathology, generating a group of 7 adenomas and 15 carcinomas. LWM-PTP expression was determined as in Figure 1. ♦ : significantly different when compared with normal paired mucosa, $p = 0.0047$.

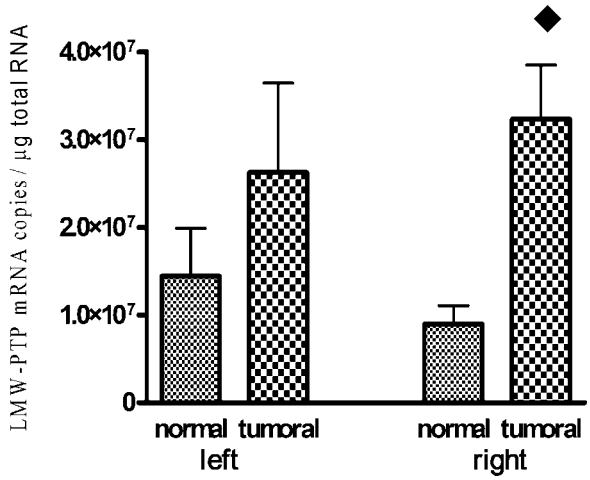

FIGURE 3 – Samples were sorted according to their localization, left-distal ($n = 12$) or proximal-right ($n = 10$). LWM-PTP expression was determined as in Figure 1. ♦ : significantly different when compared with normal paired mucosa, $p = 0.001$.

To generate the reference curve, we used cDNA derived from plasmids containing rat LMW-PTP cDNA. One microgram of the plasmid was linearised and transcribed to RNA using Ribo-Max® (Promega, Madison, WI). A standard curve was obtained by serial dilution, from 3×10^7 to 3×10^2 copies. To evaluate LMW-PTP mRNA expression in unknown samples, 400 ng of total RNA were reverse-transcribed in 80 µl of final volume in a reaction mixture containing 10 µl TaqMan® RT buffer, 5.5 mM $MgCl_2$, 500 µM each dNTPs, 2.5 µM random hexamers, 0.4 U/µl RNase inhibitors and 1.25 U/µl MultiScribe reverse transcriptase (Applied Biosystems, CA). The real time PCR reaction was performed with 25 ng/5 µl cDNA, in a reaction mix containing 300 nM of each primer, 12.5 µl Universal Master Mix and 200 nM of fluorescent probe. Plates were treated for 2′ at 50°C, 10′ at 95°C and then submitted to 40 cycles of amplification at 95°C for 15 sec, 60°C for 60 sec in the ABI Prism 7700 Sequence Detector (Applied Biosystems, CA). All samples were run in duplicate. Results were expressed as mRNA copies/µg total RNA. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA expression was also tested in each sample to verify the RNA integrity.

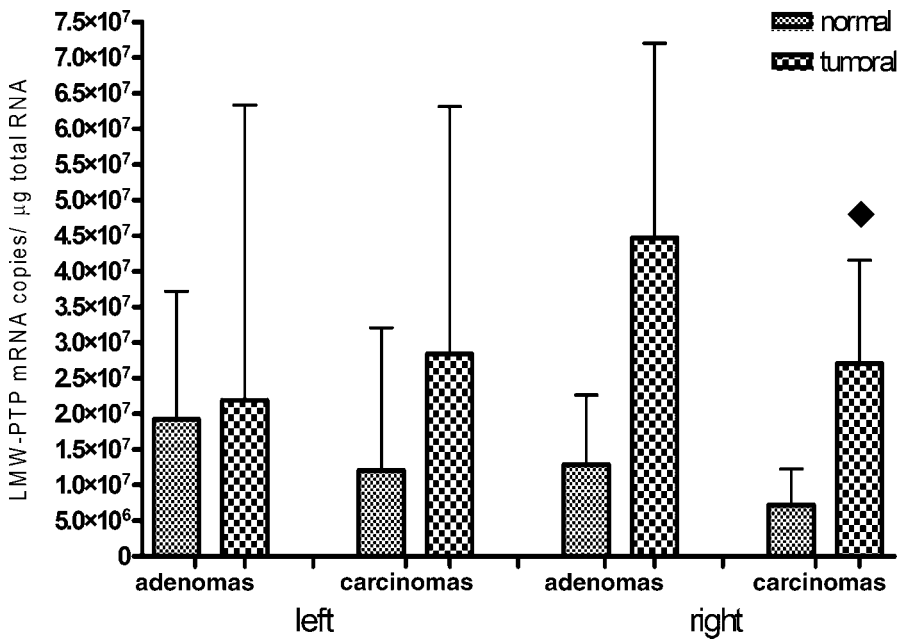

FIGURE 4 – LMW-PTP expression in cancer tissue and paired normal mucosa was sorted according to localization and histopathology. LMW-PTP expression was determined as in Figure 1. ♦ : significantly different when compared with normal paired mucosa, $p = 0.007$.

Statistical evaluation

Data obtained from the different samples were summarized for quantitative continuous responses by calculating group means and standard deviations and errors. Differences in expression between tumours and their paired normal mucosa were evaluated with Student's *t* test for paired samples. Values of $p < 0.05$ were considered significant.

Results

Colon tumours ($n = 22$) were induced in rats by treatment with DMH. The histopathological analysis of these tumours showed that 7 of them were adenomas and fifteen adenocarcinomas. The 7 adenomas were all of tubular type, six of them showed severe dysplasia, while the dysplasia in one was moderate. Twelve of the adenocarcinomas still retained a high grade of differentiation, while the differentiation was moderate in three. The level of invasion in 10 tumours was confined to the mucosa, in 3 to the submucosa, while in 2 tumours it was not possible to evaluate this parameter. The expression of LMW-PTP was analyzed in 22 samples of colonic tumours together with their apparently paired normal mucosa. The results revealed a significant ($p = 0.0016$) overall increase in LMW-PTP expression of about 2.5-fold in tumour samples as compared with their paired normal mucosa (Fig. 1).

To evaluate whether there was a difference in the expression of LMW-PTP according to the tumour histological type, the samples were divided into adenomas and adenocarcinomas, generating 2 groups of 7 adenomas and 15 adenocarcinomas. This analysis shows that the nearly 3-fold LMW-PTP increase is highly significant in adenocarcinoma samples ($p = 0.0047$), while the 2-fold increase observed in the adenoma samples did not attain statistical significance ($p = 0.1834$) (Fig. 2).

Since we already had some hints that LMW-PTP over-expression may be different in distinct locations of the colon,[12] the colonic tumours were also sorted according to location, with 12 tumour samples originating in the left distal side and 10 from the right proximal site. The 22 tumours analyzed were evenly distributed on the right and left side of the colon, with 3 adenomas and 8 carcinomas on the left side and 3 adenomas and 7 carcinomas on the right side. The results (Fig. 3) show that while the 3-fold difference in LMW-PTP expression between tumour samples and paired adjacent tissue was highly significant for right side lesions ($p = 0.001$), this was not the case for left side tumours, although a 2-fold increase can be observed also in these tumour samples. Interestingly, when sorting the tumours according to their location (left or right) and their type (adenomas and carcinomas) we observed (Fig. 4) that while carcinomas in both locations show increased LMW-PTP expression (and, notably, attaining statistical significance at the right site, with $p = 0.007$), only the adenomas located on the right side were different from normal mucosa, although with no statistical significance.

Discussion

The malignant transformation of colorectal epithelium into cancer is a multistage process, leading to adenoma and sequentially to carcinoma formation. The evolution of colorectal cancer is thought to proceed on the basis of accumulation of genetic alterations including not only mutations in various genes such as APC or K-ras but also epigenetic events.[15,16]

In a previous work we observed that LMW-PTP overexpression in human colon cancer was significantly correlated with unfavorable predictive markers such as lymphnode involvement, advanced Duke's stage, lower differentiation and un unfavorable outcome.[12] In the present work we observe a significant increase in the expression of LMW-PTP in colonic tumours induced in rats by DMH. Since within this experimental model, tumourigenesis evolves through a sequence similar to that observed in humans, *i.e.* through the sequential formation of adenomas and carcinomas, it was also possible to study LMW-PTP in the various phases of the process. The results indicate that there was a significant increase in LMW-PTP expression in adenocarcinomas, but that the 2-fold increase in the expression observed also in adenomas, did not attain statistical significance. These results suggest that the overexpression of LMW-PTP is a phenomenon associated with the onset of malignancy. Notably, when we classified the tumours according to their location, *i.e.* whether they were formed in the distal (left colon) or in the proximal (right) part of the colon, we found that there was a significant increase in the expression of LMW-PTP only in the proximal tumours when compared with the normal mucosa, while this effect was not significant in the distal tumours. Moreover, the results obtained sorting the tumours according to their location and their type, show that the most significant effect in term of increase of LMW-PTP expression is

observed in the carcinomas located at the right site. It is interesting to note that in the previous report on human colon cancers we observed a significant increase in LMW-PTP in both left and right tumours, but the expression in the right tumours was higher than that observed in those originating in the left colon.[12] These previous results together with the present ones are thus in line with several reports suggesting that right and left colon cancers are different entities with distinct molecular and cellular characteristics, a different etiology and sensitivity to chemotherapy.[17–20]

The present study may contribute to a better molecular-based classification of CRC, LMW-PTP overexpression being an additional marker of malignancy, according to our previous results in human colon cancer. These results also suggest that CRC tumours of right and left origin might be 2 partially distinct forms of cancer.

Our previous results indicate ephrin A2 receptor (EphA2) as a LMW-PTP target that may be responsible for oncogenic action due to the overexpression of tyrosine phosphatase.[9,11] In fact, EphA2 tyrosine dephosphorylation has been associated with several different human cancers.[21]

On these basis it is possible to speculate that LMW-PTP overexpression in adenocarcinomas and consequent EphA2 tyrosine dephosphorylation may be one of the effect responsible for adenocarcinoma development. Nevertheless, it cannot be excluded that other unknown substrates may exert important tumourigenic actions following their LMW-PTP-dependent tyrosine dephosphorylation. For this reason further analysis of the targets of LMW-PTP action may further contribute to understanding of its role in the development of colon cancer.

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