

The expression of leucine-rich repeat gene family members in colorectal cancer

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Abstract

This study was conducted to evaluate the association of the leucine-rich repeat (LRR) gene family with colorectal cancer (CRC). The expression of members of the LRR gene family were analyzed in 17 CRC specimens and in 59 healthy colorectal tissues by using Human Exon1.0ST microarray, and in 25 CRC specimens and 32 healthy colorectal tissues by U133Plus2.0 microarray. An association was found for 25 genes belonging to the plant-specific (PS) class of LRR genes ($P = 0.05$ for Exon1.0 ST and $P = 0.04$ for U133Plus2.0). In both data-sets, in CRC, we found down-regulation of *SHOC2* ($P < 0.00003$) and *LRRC28* ($P < 0.01$) and up-regulation of *LRSAM1* ($P < 0.000001$), while up-regulation of *MFHAS1* ($P = 0.0005$) and down-regulation of *WDFY3* ($P = 0.026$) were found only in the Exon1.0 ST data-set. The PS LRR gene class encodes proteins that activate immune cells and might play a key role in programmed cell death and autophagy. *SHOC2* and *LRRC28* genes involved in RAS-mediated signaling, which hinders nutrient deprivation-induced autophagy, might be a possible link between the negative control of autophagy and tumorigenesis.

Keywords: colorectal cancer, inflammation, autophagy, bioinformatics

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Introduction

Clinical and epidemiological studies have expanded the concept that infection and chronic inflammation are involved in tumorigenesis.¹ This is also true for onset of colorectal tumor, whose incidence is considerably increased in chronic ulcerative colitis.² Moreover, the tissue of most sporadic colorectal cancers (CRC) is largely represented by a florid stromal component with different types of inflammatory cells.³ The tumor microenvironment influences early and late stages of neoplastic progression in different ways.⁴ In early stages, an inflammatory response is triggered by the innate immune system, resulting in the release of inflammatory cytokines that enhance tumor growth.⁴ It is believed that members of the leucine-rich repeat (LRR) family play an essential role in initiating this innate immune response.⁵ So far, 34 LRR proteins of the ~400 members of this protein family have been consistently involved in pathophysiological mechanisms associated with

specific diseases, such as Toll-like receptors in innate and adaptive immunity disease⁶ and NOD (Nucleotide-binding Oligomerization Domain)-like receptors in inflammatory bowel disease.⁷ Recently, Ng *et al.*⁸ and Xavier⁹ have categorized human LRR proteins into seven classes (S, bacterial; RI, ribonuclease inhibitor-like; CC, cysteine-containing; SDS22; PS, plant-specific; T, typical and Tp, *Treponema pallidum*) based on computational and functional analyses. Based on this approach, they also characterized the function of some LRR genes including the *MFHAS1* gene, which was proposed to regulate Toll-like receptor-dependent signaling with a potential role as a negative modulator of the inflammatory response;^{8,9} the *LRSAM1* gene, a component of the antibacterial response, and the *WDFY3* gene, involved in the degradation of protein aggregates, both playing a crucial role in the autophagy process.^{8,10}

Armed with this new classification of the LRR protein family members, we intended to investigate the

contribution of members of each class to the pathogenesis of CRC using microarray approaches.

Materials and methods

Data-set description

Two microarray gene expression data-sets of tumor versus normal human colon tissues were analyzed in our study. The first data-set was composed of 42 specimens of normal mucosa and 17 paired tumor-normal colorectal tissue samples, profiled by using the Affymetrix GeneChip Human Exon 1.0 ST microarrays (Affymetrix, Santa Clara, CA, USA).^{11,12} The second data-set was composed of 32 normal and 25 CRC specimens, profiled by using Affymetrix U133Plus2.0.¹³ The data-sets were normalized by using the robust multi-array average procedure.

RNA isolation and microarray assay

The tissues prospectively collected during colonoscopy were used for RNA preparation, hybridization, staining and scanning of the microarrays performed according to the manufacturer's instructions (Affymetrix). The study was performed with the institutional review board approval and the written informed consent of all patients. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki.

Gene sets and statistical analysis

We focused our attention on the human 375 'LRR-containing protein' set described by Ng *et al.*⁸ by searching in both data-sets for the corresponding genes. A paired *t*-test was used to assess the statistical significance of expression difference for each gene.

Results

Categorization into seven regular classes of 'LRR-containing proteins'

We first investigated the gene expression levels of LRR genes, using Affymetrix Human Exon1.0 ST microarrays, in 17 CRC specimens in comparison with all 59 specimens of normal colorectal mucosa. Successively, these expression results were compared with those obtained, with a similar statistical approach but with a different microarray platform (Affymetrix U133Plus2.0), from a second cohort of 25 CRC specimens and 32 samples of normal mucosa.

Probe sets for 315 LRR genes were available in both microarray platforms, 120 of which (38%) showed significant expression changes in CRC specimens in comparison with normal mucosa (paired *t*-test, *P* = 0.05 for Exon1.0 ST and *P* = 0.04 for U133Plus2.0). The majority of altered genes (115; 96%) were dysregulated in the same direction in the two platforms (59 up- and 56 down-regulated). The PS class of LLR genes was the most enriched since ~70% of the genes belonging to this subfamily were found to be significantly dysregulated in both array platforms (Table 1).

Table 1 Genes categorized into seven regular classes of 'LRR-containing proteins' in Exon 1.0 ST and U133Plus2.0 data-sets

Classes*	LRR genes	Exon 1.0 ST	Deregulated genes		P value	Up		Down		P value	Up		Down				
			N	%		N	%	N	%		N	%	N	%			
CC	15	15	8	53.3	0.384	5	62.5	3	37.5	15	9	60.0	0.551	6	66.7	3	33.3
Mixed	14	13	7	53.8	0.392	2	28.6	5	71.4	13	9	69.2	0.298	4	44.4	5	55.6
PS	31	25	16	64.0	0.051	8	50.0	8	50.0	25	18	72.0	0.044	9	50.0	9	50.0
RI	32	30	14	46.7	0.56	8	57.1	6	42.9	30	16	53.3	0.774	10	62.5	6	37.5
S+T	60	53	19	35.8	0.967	13	68.4	6	31.6	53	32	60.4	0.416	17	53.1	15	46.9
SDS2	11	6	3	50.0	0.586	2	66.7	1	33.3	6	3	50.0	0.796	2	66.7	1	33.3
T	48	43	19	44.2	0.68	6	31.6	13	68.4	43	20	46.5	0.965	10	50.0	10	50.0
Unclassified	164	130	60	46.2	0.568	31	51.7	29	48.3	130	75	57.7	0.594	32	42.7	43	57.3
	375	315	146			75		71		315	182			90		92	

*375 human leucine-rich repeat (LRR)-containing proteins in seven classes: S, bacterial; RI, ribonuclease inhibitor-like; CC, cysteine-containing; SDS2; PS, plant-specific; T, typical; Tp, *Treponema pallidum*
P values < 0.05 are shown in bold

Table 2 Genes of category deregulated in our gene expression profile data-sets

Classes	Gene ID	Exon1.0ST			U133Plus2.0		
		P value	FDR	Regulation	P value	FDR	Regulation
PS	ERBB2IP	0.000001	0.000003	↓	0.000003	0.000004	↓
PS	LRCH1	<0.000001	<0.000001	↑	0.00002	0.00002	↑
PS	LRCH3	0.04	0.03	↓	—	—	—
PS	LRDD	—	—	—	0.000001	0.000002	↑
PS	LRRC1	<0.000001	<0.000001	↓	<0.000001	<0.000001	↓
PS	LRRC18	0.007	0.007	↓	0.000008	0.000009	↓
PS	LRRC2	0.001	0.002	↑	0.01	0.008	↑
PS	LRRC28	0.01	0.01	↓	<0.000001	<0.000001	↓
PS	LRRC39	—	—	—	0.01	0.007	↓
PS	LRRC47	0.0001	0.0002	↑	<0.000001	<0.000001	↑
PS	LRRC57	0.02	0.02	↓	0.01	0.006	↓
PS	LRRC58	0.00002	0.00004	↑	<0.000001	<0.000001	↑
PS	LRRC7	<0.000001	<0.000001	↓	0.007	0.004	↓
PS	LRRC8A	<0.000001	<0.000001	↑	0.0001	0.0001	↑
PS	LRRC8B	—	—	—	0.0003	0.0002	↑
PS	LRRC8D	—	—	—	0.0002	0.0002	↓
PS	LRRC8E	<0.000001	<0.000001	↑	<0.000001	<0.000001	↑
PS	MFHAS1	0.0005	0.0007	↑	—	—	—
PS	SCRIB	0.000005	0.00001	↑	0.000006	0.000007	↑
PS	SHOC2	0.000006	0.00001	↓	0.00003	0.00003	↓
Mixed	LRSAM1	<0.000001	0.000001	↑	0.000001	0.000001	↑
Unclassified	WDFY3	0.03	0.02	↓	—	—	—

PS, plant-specific; FDR, false discovery rate; ↓, down-regulated; ↑, up-regulated

Data were obtained comparing colorectal cancer specimens and normal adjacent tissues. Significant *P* values <0.05

Genes of LRR-PS class deregulated in our gene expression profile data-sets

After evaluating the genes deregulated in the PS class, we found that 10 of them (ERBB2IP, LRCH3, LRDD, LRRC47, LRRC57, LRRC8D, LRRC8B, MFHAS1, SCRIB and SHOC2) were described as LRR genes exhibiting elevated expression in immune tissues⁸ (Table 2 and Figure 1).

Surprisingly, two other genes recently characterized by Ng *et al.*⁸ were found significantly dysregulated in our CRCs specimens: WDFY3 and LRSAM1, presently categorized in unclassified and mixed classes, respectively (Table 2 and Figure 1).

Discussion

In this study, we investigated the expression of genes coding for members of seven classes of LRR-containing proteins in CRC specimens. Although Ng *et al.*⁸ elegantly examined the gene expression of human LRR-containing proteins into commercial human tissues, their analysis lacked information on normal or tumoral colonic tissue.¹⁴

In our study, an association was found between CRC tissues and the PS class of LRR genes, coding for proteins that recognize distinct microbial components and directly activate immune cells. Chronic inflammation caused by persistent infection with parasites, bacteria or viruses is a major driving force in tumor development.¹

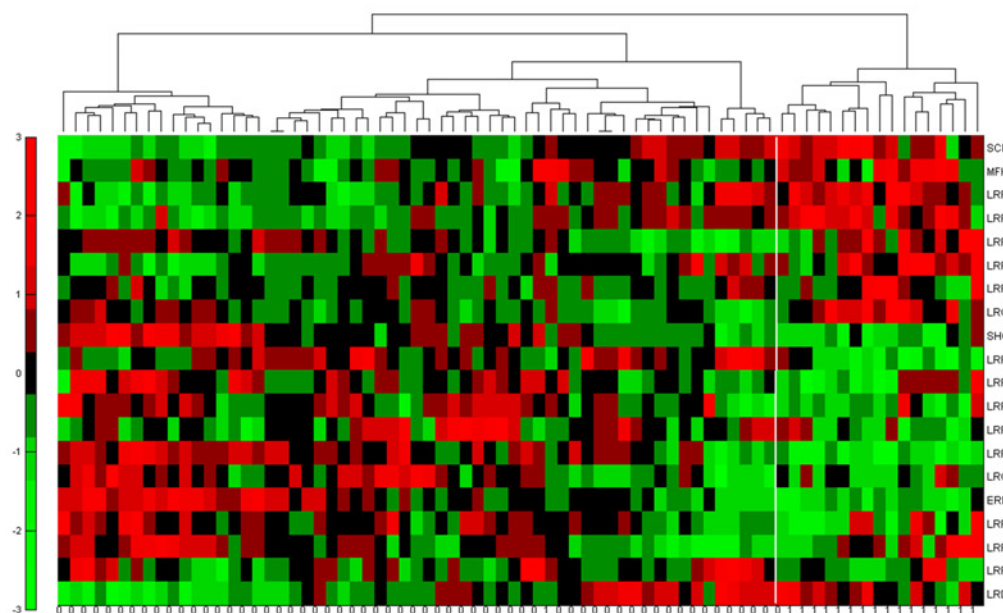
The PS LRR gene class might play a key role in host defense responses, including programmed cell death and autophagy.¹⁵ Interestingly, two genes (WDFY3 and LRSAM1) found to play a crucial role in the autophagy process⁸ were down-regulated in our study. WDFY3, also known as Autophagy-Linked FYVE protein (Alfy),

functions as a scaffold that brings together the E3 ubiquitin-protein ligase and autophagic effectors (Atg5-Atg12-Atg16L and LC3) to the target substrate to permit the degradation of expanded polyglutamine proteins in different cell types, including neurons.¹⁶ LRSAM1 is an E3 ubiquitin-protein ligase implicated in metabolism by ubiquitination of TSG101, which is a tumor suppressor gene with a reported role in maturation of the human immunodeficiency virus.¹⁷

Through literature mining, we noted that some of PS LRR genes were implicated in neoplastic diseases. The LRR-motif of MFHAS1 protein, also known as MFH-amplified sequences with leucine-rich tandem repeats 1 (MASL1), is enhanced in some malignant fibrous histiocytomas (MFH)¹⁸ and is involved in the interaction of proteins related to the cell cycle.¹⁹ SHOC2^{20,19} and LRRC28²¹ genes are involved in RAS-mediated signaling. SHOC2 positively regulates RAS/ERK mitogen-activated protein kinase (MAPK) signaling by serving as a scaffold for RAS and RAF.²² Constitutional activation of the RAS/MAPK pathway causes overlapping syndromes, comprising characteristic facial features, cardiac defects, cutaneous abnormalities, growth deficits, neurocognitive delay and predisposition to malignancies.²¹ Over-expression of LRRC28, which is involved in protein-protein interactions and signal transduction, was observed in neoplastic diseases, such as hepatocellular carcinoma.²⁰

The hampering of autophagy may benefit the survival of some tumors.²³ A negative regulator of autophagy is represented by the RAS protein, which hinders nutrient deprivation-induced autophagy through phosphatidylinositol 3-kinase signaling pathway.²⁴ In our study, SHOC2 and LRRC28 genes were found down-regulated in a statistically significant way in both CRC data-sets. This is in

(a)



(b)

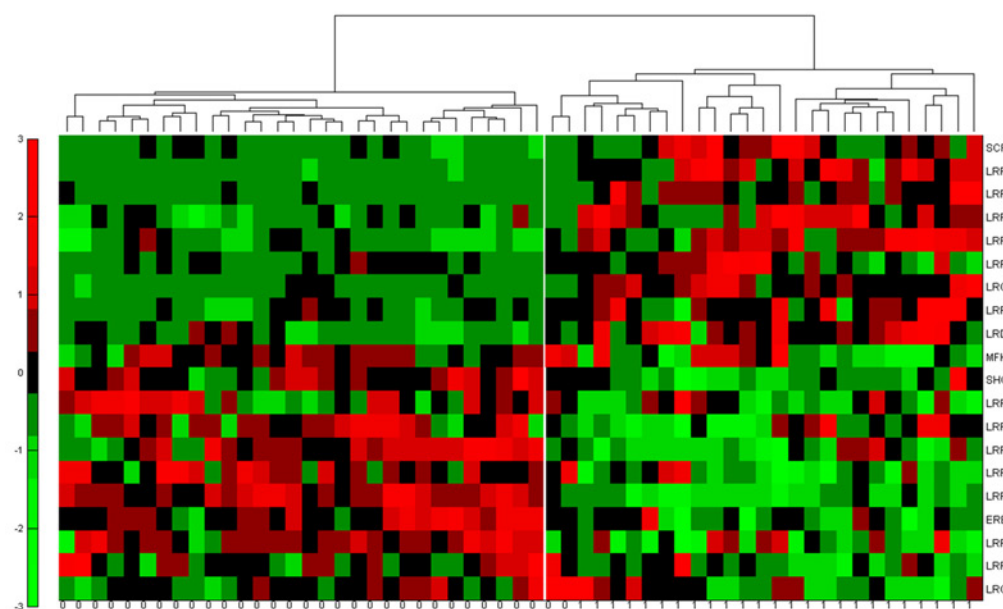


Figure 1 Hierarchical clustering of the expression of plant-specific (PS) leucine-rich repeat (LRR) genes. Expression levels of the 20 PS-LRR genes (described in Table 2) in colorectal cancer (CRC) specimens and healthy controls in the Exon1.0ST (a) and U133Plus2.0 (b) arrays data-sets are shown in heat maps. Actual sample labels are shown at the bottom of each heat map (0 = normal samples; 1 = CRC); the groups identified by hierarchical clustering analysis are separated by vertical white lines (a dendrogram is shown). (A color version of this figure is available in the online journal)

accordance with the hypothesis of Furuta *et al.*²⁴ but in disagreement with data showed by Liu *et al.*,²¹ probably in relationship to different pathogenetic mechanisms of organ-specific cancerogenesis.

Moreover, SHOC2 also interacts with *Erbin* (ERBB2IP/LAP2), another PS-LRR family gene involved in carcinogenesis.²⁵ In particular, ERBB2IP is linked to cell adhesion and the integrity or re-modeling of extracellular matrix.²⁶ Other

PS-LRR proteins, such as the LRRC7, Scribble (SCRIB/LAP4) and LRRC8 protein family, play a key role in the establishment of cell polarity, in direct or indirect cell-cell communication as well as in disruption of Ras-Raf complexes and/or in the regulation of the MAPK cascade.²⁵ In addition, the expression level of LRRC8E was found to be increased in some tumours²⁵ and SCRIB deregulation was found strongly correlated with poor survival in human

prostate cancer.²⁷ Among the PS-LRR proteins involved in carcinogenesis, the LRCHs display specific features of cytoskeletal scaffolding proteins and their failure to coordinate cell shape transformations can lead to aneuploidy,²⁸ whereas *LRRC47* genomic deletions were found to be correlated to malignant brain cancer.²⁹

The results obtained in our study are very encouraging as there is high concordance between two distinct Affymetrix platforms, supplying a direct confirmatory assay.³⁰ Indeed, the Affymetrix U133Plus2.0 array, a conventional 3' expression arrays, utilizes primers targeting the poly-A tail of mRNAs as amplification strategy, whereas the Affymetrix Exon1.0ST array employs a completely different whole transcriptome amplification process.³¹ Furthermore, the association found between the PS-LRR protein family and CRC seems specific of this neoplastic disease, also considering that in our previous study, in inflammatory bowel disease (IBD), we found an association with a totally different class of LRR protein family, and precisely LRR-mixed genes.³²

In conclusion, this study supports the idea that LRR-PS genes are deregulated in CRC specimens and might have a role in the CRC tumorigenesis. We described a close link between the negative control of autophagy and some types of tumor progression, but it needs to be confirmed by functional experiments.

Author contributions: All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript. APi, OP and G Mazzocchi wrote the manuscript; APa, EC, AG and MC designed and performed the microarray Affymetrix experiment; RM, EL and NA conducted the statistical analysis; and AL, G Marra and AA collected the biological samples.

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