Original articles

Over-representation of *PPARγ* sequence variants in sporadic cases of glioblastoma multiforme: preliminary evidence for common low penetrance modifiers for brain tumour risk in the general population

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Abstract

PPARγ, the gamma isoform of a family of peroxisome proliferator activated receptors, plays a key role in adipocyte differentiation. Recently, its broad expression in multiple tissues and several epithelial cancers has been shown. Further, somatic loss of function mutations in *PPARγ* have been found in primary colorectal carcinomas. We sought to determine if somatic high penetrance mutations in this gene might also play a role in glioblastoma multiforme (GBM). We also examined this gene to determine if common low penetrance polymorphic alleles might lend low level susceptibility to GBM in the general population. No somatic high penetrance mutations were detected in 96 sporadic GBMs. However, polymorphic alleles at codons 12 and 449 were significantly over-represented among the 27 unrelated American patients with sporadic GBM compared to 80 race matched controls. While nine (33%) were heterozygous for the P12A variant, c.34C/G (cytosine to guanine change at nucleotide 34), 12 (15%) controls were heterozygous for P12A ($p<0.05$). Similarly, 13 of 26 (50%) glioblastoma patients compared to 10 of 80 (12%) normal controls were found to have the heterozygous H449H polymorphism ($p<0.001$). The over-representation of H449H in glioblastoma patients was confirmed with a second validation set of American patients. When both American series were combined, polymorphic H449H was over-represented among cases versus controls ($p<0.001$) and there was a similar trend ($p=0.07$) for P12A. The precise mechanism for this association is unknown but these *PPARγ* polymorphisms may be acting in a low penetrance predisposing manner. However, these associations were not found in a German population, possibly arguing that if these variants are in linkage disequilibrium with a third locus, then this effect is relatively new, after the settlement of the American colonies.

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Keywords: peroxisome proliferator activated receptors; sequence variants; tumour suppressor

The gamma isoform of peroxisome proliferator activated receptor (PPARγ) is a nuclear receptor transcription factor which plays an important role in adipocyte differentiation.[1] PPARγ1 and PPARγ2 isoforms result from alternative splicing and have ligand dependent and ligand independent activation domains. It heterodimerises with retinoic acid X receptor (RXR) before it can bind ligand, after which the receptor ligand complex binds DNA and transactivates signal.[2] The full range of endogenous ligands is unknown although a prostaglandin J derivative binds to and activates this receptor.[3] The class of compounds called thiazolidinediones, of which troglitazone (Rezulin™) is a member, are synthetic ligands specific for PPARγ.[3 4] Activation of this receptor transcription factor results in a powerful adipogenic response and enhanced insulin sensitivity although pathways of differentiation in other cell types, including colon[5] and breast epithelium,[6] have been observed as well. As an important part of these differentiative responses, activation of PPARγ can lead to the slowing or cessation of cell growth.[5 7]

Recently, PPARγ has been shown to be expressed in a range of normal and neoplastic tissues, including breast and colon cancers.[5 6 8] Exposure of human breast and colon cancer lines to thiazolidinediones results in a differentiative response,[5 6] although, unexpectedly, troglitazone seemed to cause increased intestinal polyposis in the *min* mouse, which is a model of human familial adenomatous polyposis.[9 10] Interestingly, loss of function somatic *PPARγ*

Table 1 DGGE specific intronic PPARγ primers flanking each of the exons

PPARγ Exon II	259 bp*	43.52% UF†
II F: (62 nt—40 gc clamp)		
CgCCCgCCgCgCCCCgCgCCCgTCCCgCCgCCCCCgCCCg		
TTTTAACggATTgATCTTTTgC		
II R: (25 nt—5 gc clamp)		
gCgCg		
ACCCTTACATAAATGCCCCC		
PPARγ Exon 1A	205 bp	48.00% UF
1A F: (63 nt—40 gc clamp)		
CgCCCgCCgCgCCCCgCgCCCgTCCCgCCgCCCCCgCCCg		
TCTgAAACTCTgTgAgATTgCTg		
1A R: (20 nt)		
AggAgTgggAgTggTCTTCC		
PPARγ Exon 1B	283 bp	35.20% UF
1B F: (20 nt)		
CTCCgTggATCTCTCCgTAA		
1B R: (72 nt—40 gc, 10 at clamp)		
CgCCCgCCgCgCCCCgCgCCCggCCCgCCgCCCCCgCCCgAAATAATAAA		
CTgAgAgATgAggTCCAATTCT		
PPARγ Exon 2A	284 bp	42.24% UF
2A F: (70 nt—40 gc, 10 at clamp)		
CgCCCgCCgCgCCCCgCgCCCggCCCgCCgCCCCCgCCCgAAATAATAAA		
TTgCCCTgTTgCCTTTTTAg		
2A R: (20 nt)		
gCATCCTTCACAAgCATgAA		
PPARγ Exon 2B	187 bp	32.64% UF
2B F: (22 nt—2 gc clamp)		
Cg		
TCATggCAATTgAATgTCgT		
2B R: (60 nt—40 gc clamp)		
CgCCCgCCgCgCCCCgCgCCCgTCCCgCCgCCCCCgCCCg		
ggggTTCTgCTgAAATgAAA		
PPARγ Exon 3	318 bp	35.84% UF
3 F: (25 nt—5 gc clamp)		
gCgCg		
TAggTTgCTgCTTCCATgTg		
3 R: (70 nt—40 gc, 10 at clamp)		
CgCCCgCCgCgCCCCgCgCCCggCCCgCCgCCCCCgCCCgAAATAATAAA		
TCCACTggTCTggCAgCTAT		
PPARγ Exon 4A	241 bp	54.4% UF
4A F: (70 nt—40 gc, 10 at clamp)		
CgCCCgCCgCgCCCCgCgCCCggCCCgCCgCCCCCgCCCgAAATAATAAA		
gggCTTCTACTgTgTgggAA		
4A R: (25 nt—5 gc clamp)		
gCgCg		
ACTCTggATTCAgCTggTCg		
PPARγ Exon 4B	270 bp	54.08% UF
4B F: (25 nt—5 gc clamp)		
gCgCg		
gAgAAggAgAAgCTgTTggC		
4B R: (60 nt—40 gc clamp)		
CgCCCgCCgCgCCCCgCgCCCgTCCCgCCgCCCCCgCCCg		
AAACCCAAAACAACTTCCCg		
PPARγ Exon 5A	222 bp	40.96% UF
5A F: (69 nt—40 gc, 10 at clamp)		
CgCCCgCCgCgCCCCgCgCCCggCCCgCCgCCCCCgCCCgAAATAATAAA		
ACCTgggATggCATTCACT		
5A R: (21 nt—2 gc clamp)		
gC		
ATggCCACCTCTTTgCTCT		
PPARγ Exon 5B	200 bp	58.56% UF
5B F: (25 nt—5 gc clamp)		
gCgCg		
AgTTCAAACACATCACCCCC		
5B R: (61 nt—40 gc clamp)		
CgCCCgCCgCgCCCCgCgCCCgTCCCgCCgCCCCCgCCCg		
TTggTCgTTCAAgTCAAgATT		
PPARγ Exon 5C	263 bp	45.76% UF
5C F: (60 nt—40 gc clamp)		
CgCCCgCCgCgCCCCgCgCCCgTCCCgCCgCCCCCgCCCg		
TATgCCAAAAgCATTCCTgg		
5C R: (25 nt—5 gc clamp)		
gCgCg		
gCAAACTCAAACTTgggCTC		
PPARγ Exon 5D	215 bp	37.76% UF
5D F: (20 nt)		
CgAAAgCCTTTTggTgACTT		
5D R: (70 nt—40 gc, 10 at clamp)		
CgCCCgCCgCgCCCCgCgCCCggCCCgCCgCCCCCgCCCgAAATAATAAA		
ACCATCATCCCACCCTCTTT		
PPARγ Exon 6A	307 bp	55.68% UF
6A F: (70 nt—40 gc, 10 at clamp)		
CgCCCgCCgCgCCCCgCgCCCggCCCgCCgCCCCCgCCCgAAATAATAAA		
CTgAACCCCCTgTTgTgTTT		
6A R: (20 nt)		
gAgCgggTgAAgACTCATgT		
PPARγ Exon 6B	198 bp	49.60% UF
6B F: (20 nt)		
TgCAggTgATCAAgAAgACg		
6B R: (71 nt—40 gc, 10 at clamp)		
CgCCCgCCgCgCCCCgCgCCCggCCCgCCgCCCCCgCCCgAAATAATAAA		
ggTgTCAgATTTTCCCTCAgA		

*PCR product size.
†%urea-formamide at which melting is predicted.

mutations have been found in a subset of sporadic primary human colorectal carcinomas.[11]

PPARγ has been shown to be expressed in the central nervous system (CNS).[12 13] Glioblastoma multiforme represents the most common and aggressive primary neoplasm in CNS.[14] The molecular mechanisms of GBM tumourigenesis remain poorly understood. In this study, therefore, we sought to determine if somatic *PPARγ* mutations, different from those noted in colon carcinomas, are associated with primary glioblastoma multiforme. We also sought to determine if germline sequence variants in this gene could act as low penetrance alleles predisposing to sporadic brain tumours.

Materials and methods

PATIENTS

Three sets of patients with GBM were used. The subjects of the initial testing set included 27 patients with GBM operated on at the hospitals of the Ohio State University, Columbus, OH. A confirmation set included 25 independently ascertained patients operated on at the Brigham and Women's Hospital, Boston, MA. The final set comprised non-US patients, 44 German subjects with GBM. The diagnoses were made or confirmed by neuropathologists in accordance with the WHO classification. All cases were sporadic and adult onset.

Normal controls were healthy, race matched subjects from the general population who did not have cancer or a history of cancer. In general, they were people who donated blood at the respective hospitals. Thus, 80 race matched, region matched controls originated from the United States of America and 60 from Germany.

MUTATION ANALYSIS

Genomic DNA was extracted from fresh frozen brain tumours and corresponding adjacent normal brain tissue or peripheral blood leucocytes from the cases using standard procedures.[15] Control genomic DNA originated from peripheral blood leucocytes. The genomic DNA was used as template for PCR based mutation analysis. Primers used for DGGE span all seven coding exons of the gene, exon-intron junctions, and flanking intronic sequences (table 1). The seventh exon (exon II) which lies upstream of exon 1 represents the splice variant PPARγ2. Mutation analysis was carried out in four multiplex groups comprising a total of 14 fragments (table 2).

After a hot start at 94°C for three minutes, PCR amplification of genomic DNA template was carried out for 35 cycles in a modified stepdown fashion based on a basic cycle of one minute each at 94°C, 55°C, and 72°C. In the first 10 cycles, the annealing temperature was 60°C and stepped down by 0.5°C each subsequent cycle. In the second 10 cycles, the annealing temperature was held at 55°C, but the chain extension time was stepped up two seconds per cycle. In the final round of 15 cycles, the annealing temperature was maintained at 45°C and the annealing time was one minute 30 seconds. All PCR was carried out under standard conditions (Life Technologies,

Table 2 DGGE multiplex groups and final primer concentrations

MP I		*MP II*		*MP III*		*MP IV*	
Exon	*Conc*	*Exon*	*Conc*	*Exon*	*Conc*	*Exon*	*Conc*
1B	0.75 µmol/l	3	0.625 µmol/l	2B	1.5 µmol/l	5A	0.625 µmol/l
II	0.375 µmol/l	2A	0.75 µmol/l	5D	0.5 µmol/l	1A	0.375 µmol/l
6B	0.5 µmol/l	5C	0.375 µmol/l	4B	0.375 µmol/l	4A	0.5 µmol/l
5B	0.625 µmol/l	6A	0.75 µmol/l				

Gaithersburg, MD), 1.5 mmol/l $MgCl_2$ and primer concentrations, as noted in table 2. After addition of ficoll based loading buffer, 1/5 of the reaction volume was loaded on 10% polyacrylamide gels carrying a 15-65% urea-formamide gradient in 0.5 × TAE containing a 2-9% glycerol gradient. The amplicons were electrophoresed at 60°C and 100-105 V (15 mA) for 16 hours. The fragments were visualised after staining with ethidium bromide solution for 30 minutes and UV transillumination.

Samples which showed DGGE sequence variants were subjected to reamplification using primers for DGGE or those described previously.[16] Semi-automated sequencing was performed as previously described.[17]

WESTERN BLOT ANALYSIS

Whole protein lysates were obtained from GBM cell lines A172 and U87, as previously described.[18] These two are well characterised GBM lines whose characteristics are similar to the primary tumours. Fractionation of protein, 100 µg from A172 and 150 µg from U87, through polyacrylamide gel electrophoresis, fixation on membrane, and immunoblotting with polyclonal antibody raised against PPARγ (Santa Cruz Biochemicals, Santa Cruz, CA) was performed as previously reported.[6] Protein signals were detected by enhanced chemiluminesence (Amersham, Piscataway, NJ).

STATISTICAL ANALYSIS

Differences in frequencies of alleles were calculated using the standard chi-squared or Fisher Exact Test, as previously applied.[19] The effect of genotype distribution and GBM was evaluated with odds ratios (±95% confidence intervals) comparing the heterozygous genotype to the wild type at each locus.

Results

Mutation analysis of *PPARγ* in 96 sporadic GBM tumours showed no pathogenic somatic mutations in the coding sequence, including that for exon II, and flanking intronic sequences. Two previously described polymorphisms, P12A in exon II (CCA>GCA at codon 12, c.34C/G) and H449H in exon 6 (CAC>CAT at codon 449, c.1347C/T), were noted in these patients. A novel, rare polymorphic variant resulting from a silent base substitution (ATC>ATT) at codon 456 (I456I) in exon 6 was found in a single American case.

Among a pilot set of 27 Americans with GBM, nine (33%) were heterozygous for the P12A variant, c.34C/G. In contrast, of 80 normal controls, 12 (15%) were heterozygous for P12A ($p<0.05$). No homozygosity for the variant allele was observed in our series of cases and controls. The over-representation of the polymorphic G allele in the GBM cases was also evident compared to controls (p~0.05, table 3). Similarly, 13 of 26 (50%) glioblastoma patients compared to 10 of 80 (12%) normal controls were found to have the heterozygous H449H polymorphism ($p<0.001$). The polymorphic T allele occurred 13 times among 54 total glioblastoma alleles compared to 10 out of 160 control alleles ($p<0.001$, table 3).

A second set of 25 American patients with GBM were examined for *PPARγ* polymorphism frequencies to confirm the observations in the first set of patients. Among these cases, there were six P12A alleles and 44 wild type alleles compared to 12 and 148, respectively, among controls ($p>0.05$). At codon 449, there were eight variant alleles and 42 wild type among GBM patients compared to 10 variant and 150 wild type, respectively, among controls ($p=0.03$, table 3). Similarly, cases with at least one H449H allele were over-represented in this cohort of cases (8 of 25) compared to their race matched controls (10 of 80, $p<0.05$).

Because the two sets of American cases were somewhat similar, a reanalysis was performed with the combined sets comprising a total of 52 cases of GBM. In this large cohort of brain tumour cases, there was over-representation of the H449H polymorphic allele compared to the race matched normal controls ($p<0.001$, table 3). There was a trend towards over-representation of P12A in this summative cohort as well ($p=0.07$).

To determine if over-representation of P12A or H449H or both could be generalisable to populations outside the USA, a set of 44 German glioblastoma multiforme cases were examined for allele frequencies. In contrast to the American series, there were no significant differences in allelic or genotype frequencies at codons 12 and 449 between German cases and race matched controls ($p>0.05$, table 3).

Genotypes at each locus were formed for cases and controls from both populations

Table 3 Comparison of frequencies of polymorphic alleles at codons 12 and 449 among US and German glioblastoma cases and race matched controls

Series	*Polymorphic allele*	*Wild type allele*	*Chi-squared*	*p value*
(A) P12A				
US controls	12	148	—	—
Cases - USA 1	9	45	3.83	p~0.05
Cases - USA 2	6	44	0.98	p>0.05
Cases - US 1+2	15	89	3.3	p=0.07
Cases - German	9	79	0.11	p>0.05
German controls	14	106	—	—
(B) H449H				
US controls	10	150	—	—
Cases - USA 1	13	41	13.38	p<0.001
Cases - USA 2	8	42	4.62	p=0.03
Cases - US 1+2	21	83	11.82	p<0.001
Cases - German	12	76	1.03	p>0.05
German controls	11	109	—	—

Table 4 Distribution of genotype at codon 12 and codon 446 in GBM cases versus controls

Series	c.34 C/C (WT)	c.34 C/G	c.34 G/G*	Odds ratio	95% confidence intervals
P12A (c.34 C/G)					
US controls	68	12	0	—	—
Cases - US 1	18	9	0	2.8	1.0–7.8
Cases - US 2	19	6	0	1.8	0.6–5.4
Cases - US 1+2	37	15	0	2.3	1.0–5.4
German controls	46	14	0	—	—
Cases - German	35	9	0	0.8	0.3–2.2
Series	*C/C (WT)*	*C/T*	*T/T**	*Odds ratio*	*95% confidence intervals*
H446H (c.1338C/T)					
US controls	70	10	0	—	—
Cases - US 1	14	13	0	6.5	2.4–17.7
Cases - US 2	17	8	0	3.3	1.1–9.6
Cases - US 1+2	31	21	0	4.7	2.0–11.2
German controls	49	11	0	—	—
Cases - German	33	10	1	1.35	0.5–3.5

*Because of the small number of homozygous variants at both loci, odds ratios did not take variant homozygotes into the equation (see Materials and methods).

(table 4). The differences in genotype distribution among cases and controls serve to confirm the allelic analyses. Specifically, among the combined US cases, the odds ratio for the combined US series for P12A is 2.3 (95% CI 1.0-5.4) and for H449H is 4.7 (2.0-11.2) (table 4). In contrast, the German series equivalent odds ratios were 0.8 (0.3-2.2) and 1.4 (0.5-3.5), respectively (table 4).

Because P12A lies within PPARγ2 and PPARγ2 was not believed to be expressed in brain, GBM cell lines A172 and U87 were examined for expression of the two isoforms of this protein. Western analysis using the antibody against PPARγ showed that both PPARγ1 and PPARγ2 were expressed in both lines (data not shown).

Discussion

Although no somatic pathogenic *PPARγ* mutations were found in sporadic GBM, a common sequence variant, H449H, and possibly P12A as well, were found by both allelic and genotype analyses to be significantly over-represented in American patients with sporadic GBM. Although the variant allele over-representation is not generalisable to the German GBM cases, the over-representation of polymorphic alleles in US GBM cases versus controls is probably real for several reasons. Firstly, the differences are statistically significant despite the modest size of the case series. Secondly, at least for H449H, the findings were confirmed in a second independent set of GBM cases. Thirdly, preliminary analysis of these two polymorphisms in patients with other cancer types, such as breast cancer, ovarian cancer, head and neck cancer, and melanoma, showed no over- or under-representation of polymorphic alleles in those patients (Eng *et al*, unpublished observations). This argues for the specificity of this observation. Fourthly, our normal controls were found to have similar allele frequencies at these two loci compared to the published cases.[16 20]

It is unclear why allelic over-representation is not evident in the German GBM series compared to both US series. One speculation might be that the association, although present, is not as pronounced in the German cases as the controls, and hence a much larger series of both cases and controls is necessary to note the over-representation of alleles. This is not uncommon. An alternative explanation is based on a corollary of founder effect theory.[21] Since non-coding single nucleotide polymorphisms appear to be associated with GBM, it is just as plausible that these loci are in linkage disequilibrium with a third locus, which lends low level susceptibility to GBM. If so, then it might be that the low penetrance susceptibility allele occurred only after the settlement of the United States, and so one would never see the allelic association in Europe, that is, a relatively new founding allele.

The precise mechanism and the significance underlying the over-representation of the two polymorphic alleles in patients with sporadic GBM were not immediately obvious. Since the H449H-GBM association is more robust, it might be that H446H can act as an independent low penetrance allele. Residue 449 lies within the common region found in both isoforms. A previous study showed over-representation of the polymorphic H446H allele with increased leptin levels but only in obese subjects.[20] Our patients represent the general population and were not selected for obesity. Hence, this is probably not germane. If we assume that H449H can act as an independent low penetrance susceptibility allele for glioblastoma, then several mechanisms can be invoked. A new cryptic splice donor, acceptor, or enhancer may be created by this nucleotide change, although this has not been directly observed in the past despite previous and continuing studies of variation in this gene in human populations (as above). It is possible that the sequence variant may predispose to decreased expression of the variant bearing allele, thus leading to low level functional haploinsufficiency. Finally, preferential usage of tRNA molecules may be hypothesised, although this has yet to be shown in humans, but is well described among prokaryotes. If this were true, the wild type triplet would be favoured by the tRNAHis while the variant would be less favoured, thus resulting in slightly decreased efficiency of PPARγ translation in the latter. This would presumably result in a low level functional haploinsufficiency. Alternatively, another locus in linkage disequilibrium with the H449H polymorphism might be the actual low penetrance susceptibility locus.

If we considered the first American set of cases and the combined American series as a whole, P12A over-representation in cases versus controls approaches significance ($p=0.05$ and $p=0.07$, respectively). Initially, the possible P12A association with GBM was puzzling from a biological point of view given that PPARγ2, on which codon 12 resides, was believed to be expressed at significant levels only in adipocytes. However, our data indicate for the first time that PPARγ2 can also be expressed in GBM cells or at least cell lines. The putative effect of P12A can be direct (organ specific) or indirect (systemic). If P12A

results in a direct, albeit low level, predisposition in brain tissue, then PPARγ must act as a tumour suppressor. Our studies of PPARγ in colorectal carcinomas support the view that it is a tumour suppressor.[5 11] Because somatic heterozygous loss of function mutations were found in these cancers, it was postulated that either a dominant negative or haploinsufficiency model could account for biallelic inactivation.[11] It is believed that the P12A isoform results in slightly decreased receptor activity.[22] Hence, subjects with P12A have PPARγ2 receptors with decreased activity, leading to low level haploinsufficiency, in the brain. Other genetic and epigenetic insults built upon this slight predisposition would then result in GBM formation. Further, it is also possible that P12A can act indirectly to contribute to low penetrance predisposition to GBM. Some investigators have found that the alanine isoform has slightly decreased receptor activity.[22] An independent clinical epidemiological study in Israel has found that high fat and cholesterol were inversely related to the development of glioneural tumours but unrelated to meningioma formation.[23] Thus, the converse might also be true; low cholesterol and low triglycerides are associated with glial tumours.

In summary, we have found an overrepresentation of the H449H polymorphism, and possibly the P12A polymorphism, in the *PPARγ* gene in American patients with sporadic glioblastoma multiforme. We believe that P12A and H449H themselves, or a third locus in linkage disequilibrium with them, might be important low penetrance susceptibility loci for glioneural tumours. However, whether this is generalisable to all populations in the world requires confirmation in a broad range of populations and in larger series. It would seem, from our limited study of a single small set of non-US patients, that this might not be generalisable to all populations, perhaps not to those of the old world. Thus, further molecular, epidemiological, and functional studies in this regard are warranted.

Note added in proof

In previous publications, codon numbering was based on a cDNA sequence which excluded three amino acids from the 5' end of exon 6. We have novel sequencing data which show the presence of the three additional codons (W M Smith, C Eng, unpublished data) and so we have adjusted the exon 6 codon numbers accordingly; therefore, H446H is now referred to as H449H.

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