Supplementary data

Supplementary Note 1: Lack of antibodies that specifically recognize AHRR

Quantitation of human AHRR (hAHRR) protein levels in tumors and normal tissue is not shown in this study due to the lack of a working AHRR antibody at the time the experiments were done.

The first reference to an AHRR antibody appeared in 2003 (1). This publication included an immunocytochemistry (IHC) for AHRR on a cryosection of pituitary using an AHRR antibody generated by Dr. Mimura. No characterization of the antibody (including negative controls for the immunocytochemistry as absorption controls or western blots to confirm the size of the immunoreactive band) were shown in this publication. Our group unsuccessfully attempted to characterize this antibody. Even though several publications have focused on AHRR expression and localization (2-7), only two have shown detection of AHRR by ICH (1, 8) and none have shown AHRR protein quantitation (by western blot, RIA or any other assay involving the use of a specific antibody for AHRR) and all report AHRR mRNA measurements instead. Therefore, evidence from previous studies and our own experience supported that an antibody for the analysis (specifically quantification by western blot, RIA, ELISA, etc) of hAHRR protein was not available at the time we performed the experiments included in this manuscript.

A new polyclonal antiAHRR antiserum has recently been reported (9). The antibody was made against the peptide CQFQGKLKFLFGQKKK. Analysis of this publication and the peptide sequence reveals the following:

1- The authors do not provide data for the characterization of their newly generated antiAHRR antibody through western blot followed by absorption controls.

2- the sequence of the peptide used for immunization is located in a region which is highly conserved and shares high homology with other members of the bHLH/PAS family of transcription factors such as the human aryl hydrocarbon receptor (hAHR) as assessed by Blast analysis:

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Identities = 12/14 (85%), Positives = 13/14 (92%), Gaps = 0/14 (0%)

Peptide used for immunization  3    FQGKLKFLFGQKKK  16
                                    FQGKLK+L GQKKK
hAHR                           239  FQGKLKYLHGQKKK  252
```

Sequence 1: hAHR Locus NP_001612 Length = 848 (1 .. 848)
Sequence 2: hAHRR Locus NP_065782 Length = 719 (1 .. 719)

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 Score =  273 bits (699),  Expect = 5e-71
 Identities = 186/413 (45%), Positives = 238/413 (57%), Gaps = 51/413 (12%)

Query  9    TYASRKRRKPVQKTVKPIPAEGIKSNPSKRHRDRLNTELDRLASLLPFPQDVINKLDKLS  68
            TYA RKRR+P+QK    + AE  KSNPSKRHRDRLN ELD LASLLPFP D+I+KLDKLS
Sbjct  12   TYAGRKRRRPLQKQRPAVGAE--KSNPSKRHRDRLNAELDHLASLLPFPPDIISKLDKLS  69

Query  69   VLRLSVSYLRAKSFFDVALKSSPTERNGGQ----DNCRAANFREGLNLQEGEFLLQALNG  124
            VLRLSVSYLR KSFF V  + S  +   G     D+C  A    G  + EG  LL++LNG
Sbjct  70   VLRLSVSYLRVKSFFQVVQEQSSRQPAAGAPSPGDSCPLA----GSAVLEGRLLLESLNG  125

Query  125  FVLVVTTDALVFYASSTIQDYLGFQQSDVIHQSVYELIHTEDRAEFQRQLHWALNPSQCT  184
            F LVV+ +  +FYAS+TI DYLGF Q+DV+HQ++Y+ IH +DR +F RQLHWA++P Q
Sbjct  126  FALVVSAEGTIFYASATIVDYLGFHQTDVMHQNIYDYIHVDDRQDFCRQLHWAMDPPQVV  185

Query  185  ESGQGIEEATGLPQTVVCYNPDQ-----IPPENSPLMERCFICRLRCLLDNSSGFLA---  236
              GQ     TG    +      Q      P E S  + RCFICR+RCLLD++SGFLA
Sbjct  186  -FGQPPPLETGDDAILGRLLRAQEWGTGTPTEYSAFLTRCFICRVRCLLDSTSGFLARGS  244

Query  237  ---------------MNFQGKLKYLHGQKKKGKDGSILPPQLALFAIATPLQPPSILEIR  281
                          M FQGKLK+L GQKKK   G++LPP+L+LF IA P+  PS  E++
Sbjct  245  QAWQLRLCCPEPLMTMQFQGKLKFLFGQKKKAPSGAMLPPRLSLFCIAAPVLLPSAAEMK  304

Query  282  TKNFIFRTKHKLDFTPIGCDAKGRIVLGYTEAELCTRGSGYQFIHAADMLYCAESHIRMI  341
             ++ + R K + D T    DAK +      E+EL  + +           Y A    R
Sbjct  305  MRSALLRAKPRAD-TAATADAKVKATTSLCESELHGKPN-----------YSAGRSSR--  350

Query  342  KTGESGMIVFRLLTKNNRWTWVQSNARLLYKNGRPDYIIVTQRPLTDEEGTEH  394
               ESG++V R  T   RW  V + A  L   G PD ++  +    D E  +H
Sbjct  351  ---ESGVLVLREQTDAGRWAQVPARAPCLCLRGGPDLVLDPKGGSGDREEEQH  400
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It seems logical to consider the possibility that this polyclonal antibody would recognize several epitopes in this peptide some of which could be found in AHR. Therefore, we feel that full characterization of this antibody is needed.

Supplementary Table 1: Primer sets covering exons 2-11 of the *AHRR* gene used in the mutational analysis.

Primer name	Sequence	Primer size
Ex2F	5'-ccaaccctccccttccat-3'	18 bp
Ex2R	5'-gtgccccctgtcttccag-3'	19 bp
Ex3F	5'-aagtgaacaggtgggagcag-3'	20 bp
Ex3R	5'-cacctgacccagaccatctc-3'	20 bp
Ex4F	5'-cggagaaccaagtgtcaagtg-3'	21 bp
Ex4R	5'-gggggtgcctaatgtgtctt-3'	20 bp
Ex5F	5'-tggagggctattctggtttc-3'	20 bp
Ex5R	5'-cattttgggtgagccaattc-3'	20 bp
Ex6F	5'-gactcacttggaccccagac-3'	20bp
Ex6R	5'-cacttggggtaaggctgaaa-3'	20bp
Ex7F	5'-agggacccacgcactcac-3'	18 bp
Ex7R	5'-ccttggcccctatggtct-3'	18 bp
Ex8F	5'-acagcggctggaattcgta-3'	19 bp
Ex8R	5'-ggggatggtttcaggatgat-3'	20 bp
Ex9F	5'-gcaccatgtggctgtgaa-3'	18 bp
Ex9R	5'-accagacgatgcagtttcaa-3'	20 bp
Ex10F	5'-cccatgtggaaaagatcaga-3'	20 bp
Ex10R	5'-tgtcatcttgttcatccgtca-3'	21 bp
Ex11F	5'-gctgcctggcaccacttac-3'	19 bp
Ex11R	5'-ccccctctaaaccccaac-3'	18 bp

Supplementary Table 2: Summary of SSCP and sequencing analysis of AHRR gene in cervical cancer (n = 30)

Exon/Tumor	SSCP Variant in tumor	Nucleotide in normal DNA	Nucleotide change in tumor	cDNA position	Comments
Ex11/T-82	+	T/G	T prominent G residual	1216 bp	T/G at 1216 is a known polymorphism; residual G peak in tumor indicates LOH of the allele containing G
Ex11/T-93	+	T/G	G prominent T residual	1216 bp	T/G at 1216 is a known polymorphism; residual T peak in tumor indicates LOH of the allele containing T
Ex4/T-104	+	G/A	G/A	385 bp	This is a polymorphism since it was present in both tumor and normal DNA. (An intronic polymorphism was also seen but not stated here)
Ex6/T-117	+	G/C	G	609 bp	Only G peak is present in Tumor; consistent with LOH of allele containing C; Known polymorphism
Ex6/CaSki	+	Not known	T→G	565 bp	This is polymorphism or not can not be ruled out as this is a cell line
Ex6/C-33A	+	Not known	T/C	508 bp	This is polymorphism or not

					can not be rule out as this is a cell line

In addition to the data shown in this table, sequencing of the AHRR expression plasmids confirmed some of the variants already described (http://www.ensembl.org/Homo_sapiens/protview?db=core;peptide=ENSP00000323816) . No conclusive data showing association of mutations or polymorphisms of *AHRR* to cancer were generated in this study. Although an association has been shown between *AHRR* polymorphism and male infertility (10, 11), micropenis (12), and advanced stage endometriosis (13), no relationship has been found with endometriosis (2) or lung cancer (14). The data presented in the this study together with the current literature do not support that mutations or polymorphisms are relevant in the biology of AHRR as it relates to carcinogenesis.

Supplementary Table 3: Reactivation of *AHRR* mRNA expression after treatment with demethylating agent.

Cancer type	*AHRR* mRNA reactivation*			
	0-1.4	1.5-2.9	3-10	>10
Cervix	3	3	2	1
Lung			2	1
Testis	1			1

* Samples were treated with 5-Aza-2' deoxycytidine, TSA or a combination of both. Results are expressed as fold increase of *AHRR* mRNA levels of cells unexposed versus exposed with the treatment that showed higher differences.

Supplementary Table 3: Transcription factor binding sites in the –333 to +27 bp of the promoter region of the *AHRR* gene

Transcription factor	OMS	MS	Position From/To	Str	Sequence
Winged helix protein, involved in hair keratinization and thymus epithelium differentiation	0.95	0.956	-333/-23	(+)	aggACGCcggt
Zinc finger transcription factor ZBP-89	0.93	0.951	-269/-247	(-)	gtcccggcctCCCCcagagaagc
Myeloid zinc finger protein MZF1	0.98	0.985	-233/-227	(+)	gaGGGGa
c-Ets-1 binding site	0.92	0.92	-142/-126	(+)	ggagcAGGAggtggggg
Basic krueppel-like factor (KLF3)	0.95	0.956	-113/-101	(+)	gcGGGTgtggggg
Wilms Tumor Suppressor	0.88	0.965	-111/-97	(+)	gggtgTGGGggcgcc
Zinc finger / POZ domain transcription factor	0.95	0.956	-105/-95	(+)	ggggGCGCcag
Upstream stimulating factor	0.86	0.986		(-)	gcgcCACGtgcgccc
Hypoxia induced factor-1 (HIF-1)	0.87	0.976		(+)	gggcgcACGTggcgc
Hypoxia inducible factor, bHLH / PAS protein family	0.93	0.967		(+)	gggcgcaCGTGgcgc
AHR nuclear translocator homodimers	0.89	0.965		(+)	gggcgcaCGTGgcgc
Upstream stimulating factor	0.92	0.936		(-)	gcgcCACGtgcgccc
MYC-MAX binding sites	0.92	0.982		(-)	gcgcCACGtgcgccc
Max	0.85	0.936		(-)	gcgcCACGtgcgccc
Upstream stimulating factor	0.91	0.985		(-)	gcgccaCGTGcgccc
N-Myc	0.91	0.979	-56/-42	(-)	gcgccaCGTGcgccc
Upstream stimulating factor	0.86	0.986	-55/-41	(+)	ggcgCACGtggcgcg
Upstream stimulating factor	0.92	0.937		(+)	ggcgCACGtggcgcg
Max	0.85	0.936		(+)	ggcgCACGtggcgcg
c-Myc/Max heterodimer	0.92	0.929		(+)	ggcgCACGtggcgcg
Upstream stimulating factor	0.91	0.955		(+)	ggcgcaCGTGgcgcg
N-Myc	0.91	0.978		(+)	ggcgcaCGTGgcgcg

Hypoxia inducible factor, bHLH / PAS protein family	0.93	0.934		(-)	cgcgccaCGTGcgcc
AHR nuclear translocator homodimers	0.89	0.943		(-)	cgcgccaCGTGcgcc
Elk-1	0.92	0.942	-47/-31	(-)	gtcaccGGAAcgcgcca
Activator protein 2	0.89	0.907	+5/+17	(+)	atCCCGccgggggg

MS: Matrix similarity; OMS: Optimized MS

Supplementary Figure 1

Evaluation of non-silencing controls. A: Comparison of AHRR mRNA levels in A549E, A549sr and A549wt. As expected no statistically significant differences were observed. B: Evaluation of the growth potential of A549E (squares), A549sr (diamonds) and A549wt (circles) over time. As expected from their similar AHRR levels no differences in the growth rate was observed. C: Comparison of the migratory potential of A549E, A549sr and A549wt. No differences in the migratory potential of the three cells lines were observed. Collectively these data supporting that the three cells lines exhibit similar phenotypes validating them for their use as non-silencing controls.

Supplementary Figure 2

Identification of promoter hypermethylation in cervical and ovarian carcinomas, and testicular germ cell tumors by methylation-specific PCR. U, unmethylated allele; M, methylated allele; C-33A, HT-3, SiHa, ME-180, HeLa, and T-16 represent cervical cancer; OVACAR29, A224, CP20, and OVT2 are ovarian cancer; T-178A, T-480A, T-407A, and T-395 are testicular germ cell tumors. All samples were run in the same gel although were noncontiguous.

Supplementary Figure 3

Real time PCR measurements of *AHRR* mRNA levels in bronchioloalveolar carcinoma A549 (a) and normal breast MCF10A (b) transfectants (experiments were run in triplicate). Cells transfected with siRNA for *AHRR* (A549F/G and MCF10A-F/G)

showed significant reduction in *AHRR* mRNA when compared with empty vector transfected cells (A549E and MCF10A-E). Notice that the effect of the siRNA seems to be more prominent in A549 than in MCF10A. *, P<0.05; **, P<0.01; ***, P<0.001

References

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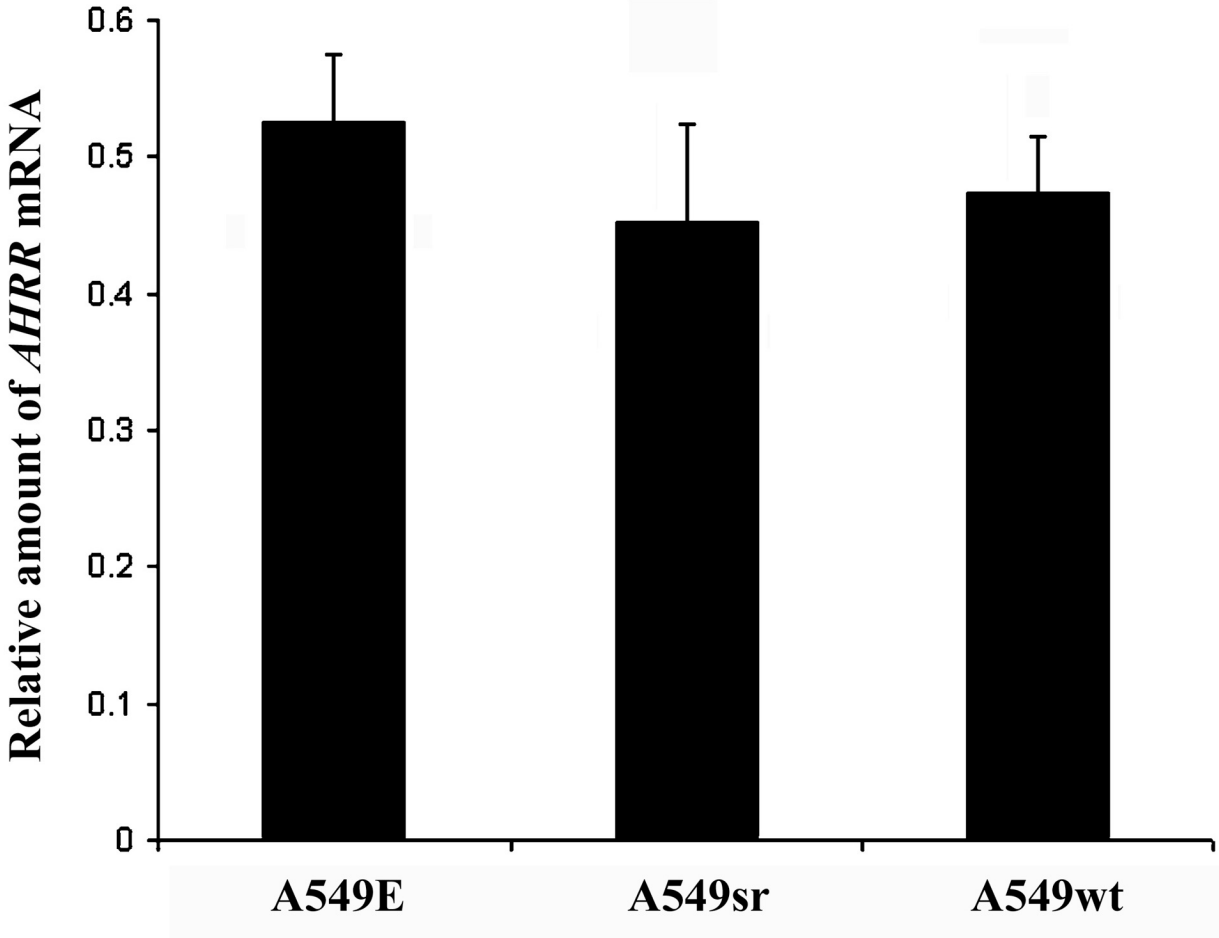
A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0.2
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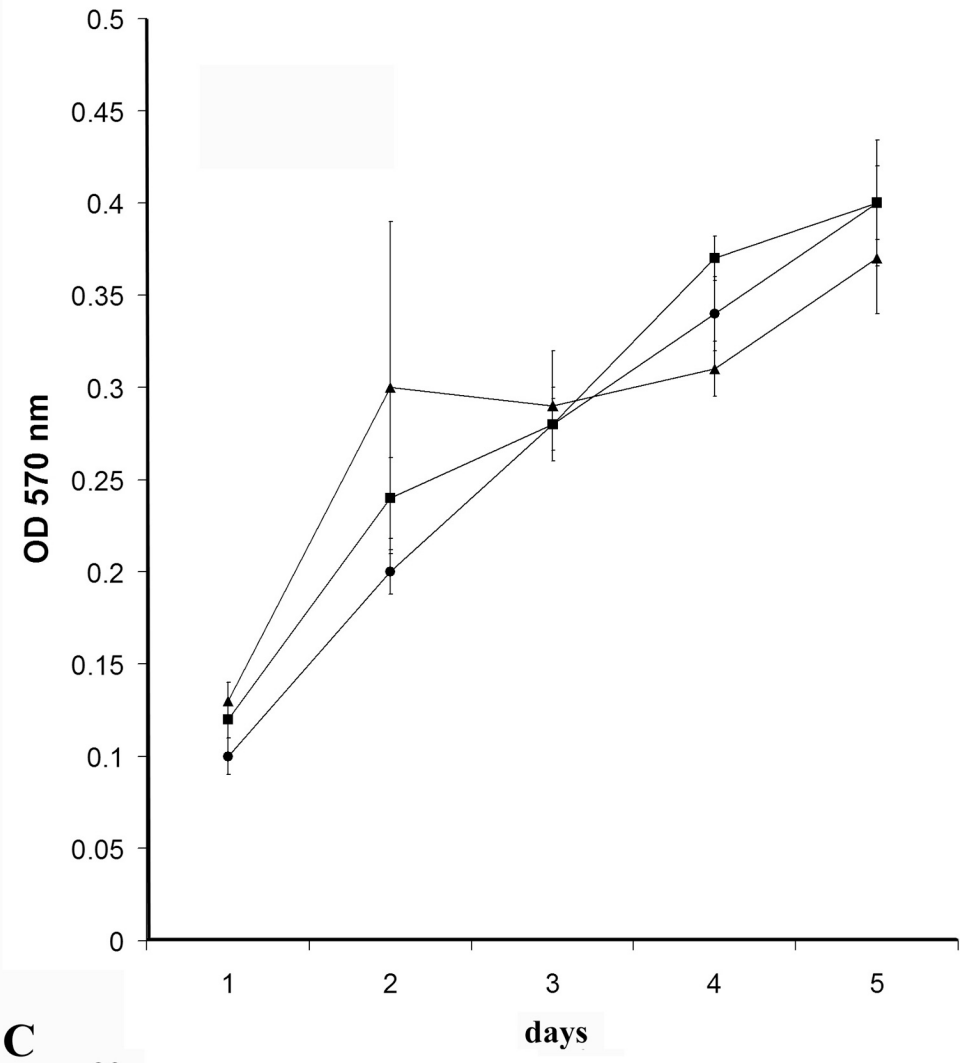
A549E
A549sr
A549wt

B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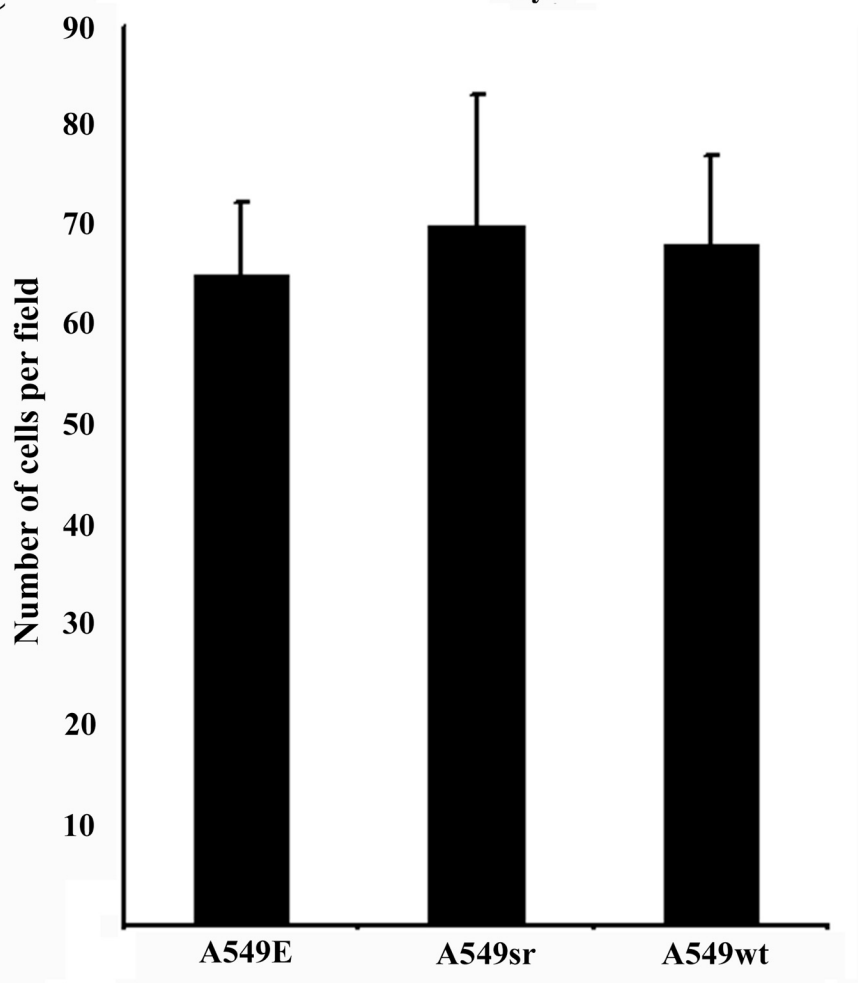
2
3
4
5
days

C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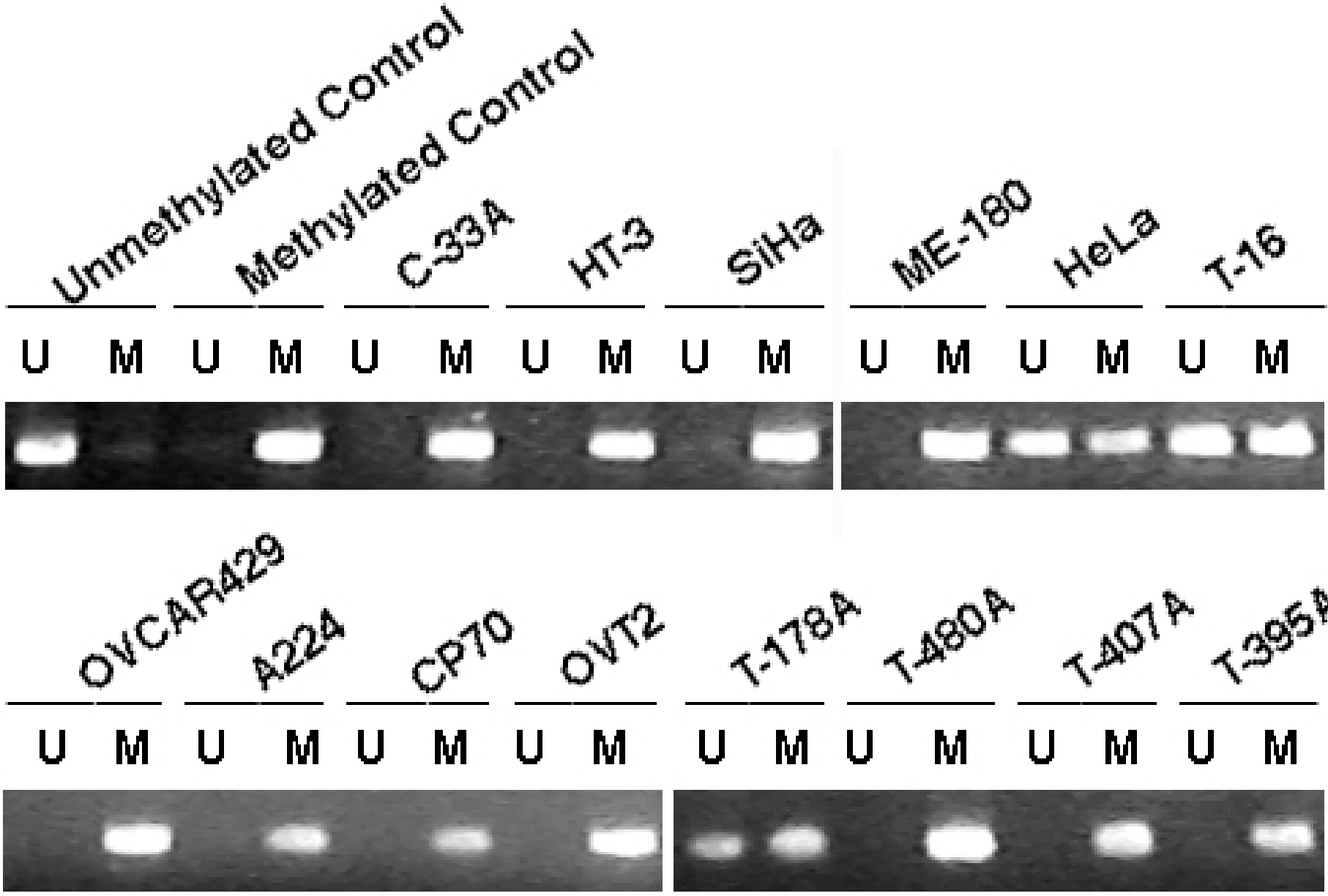
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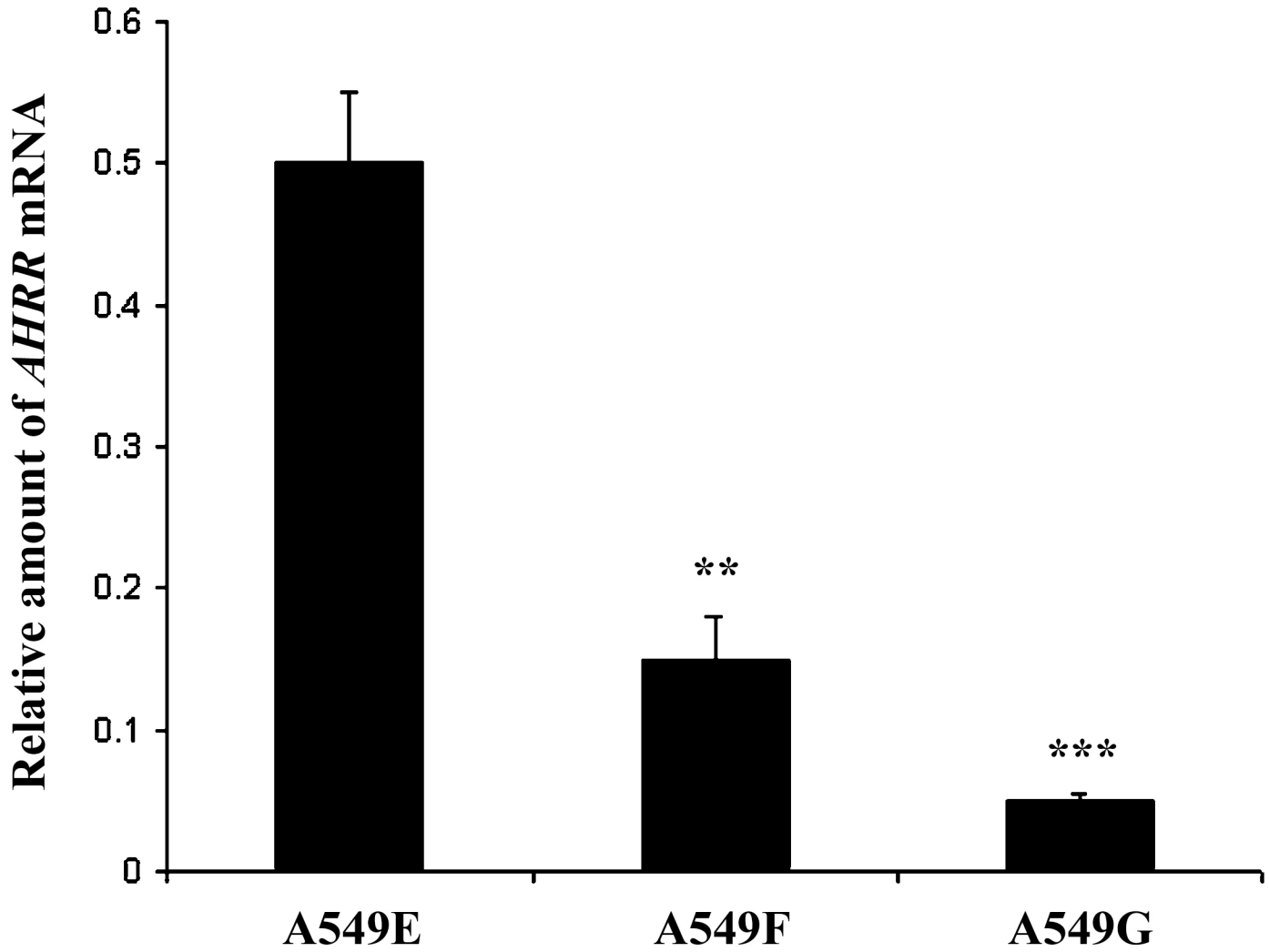
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0.4
0.3
0.2
0.1
0
**
***
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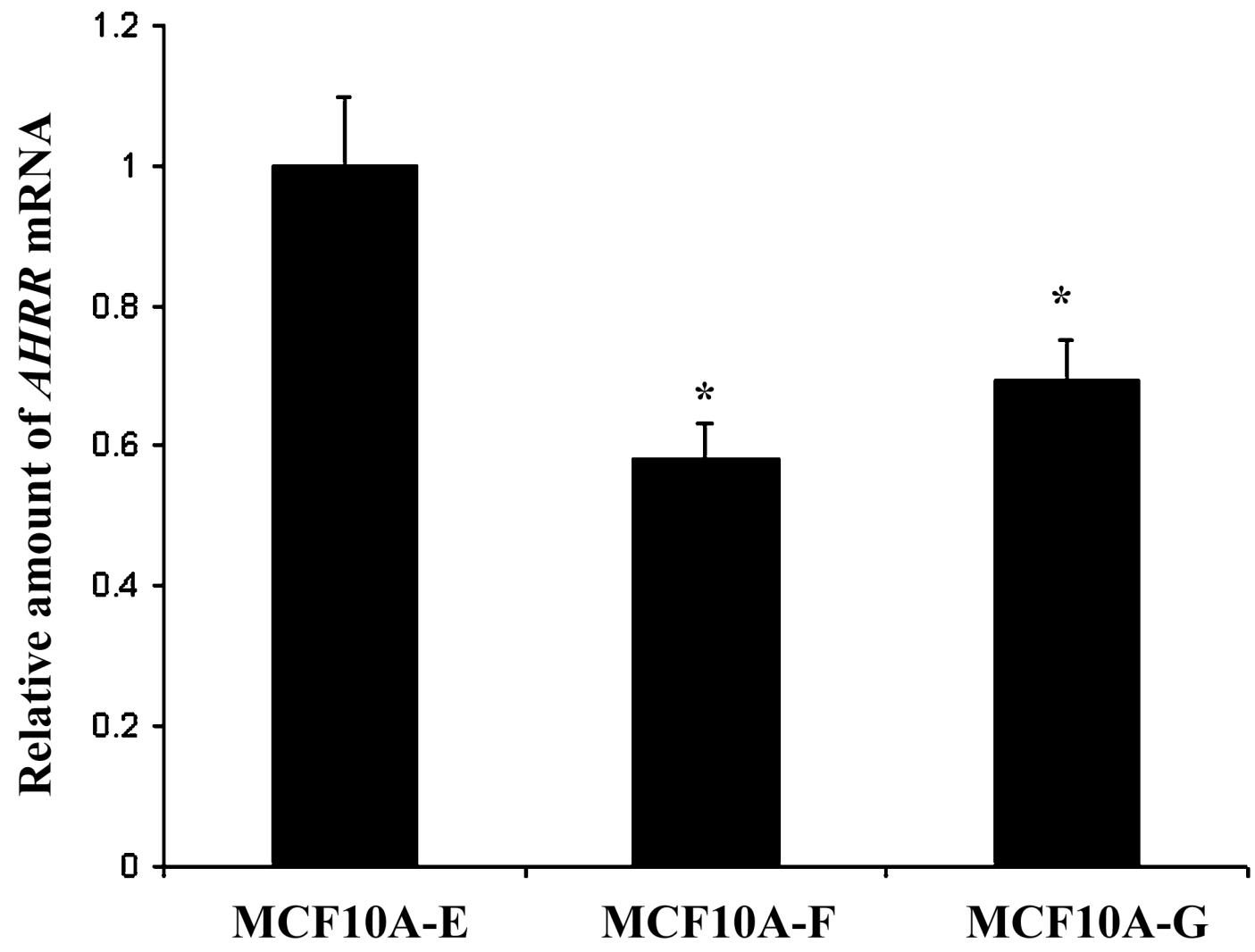
A549F
A549G

B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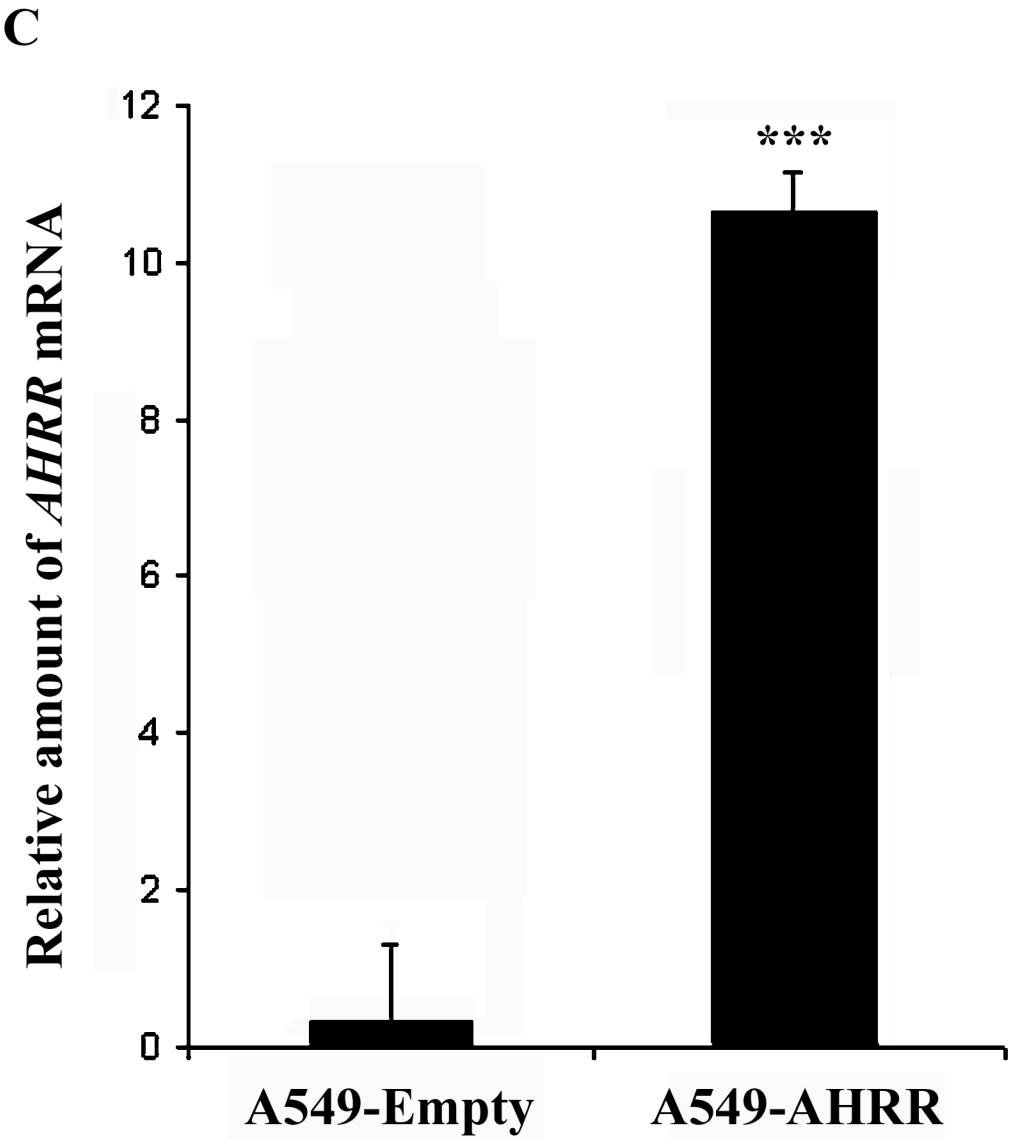
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