The aryl hydrocarbon receptor repressor (AHRR) is a putative tumor suppressor gene in multiple human cancers

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The aryl hydrocarbon receptor repressor (AHRR) is a bHLH/Per-ARNT-Sim transcription factor located in a region of chromosome 5 (5p15.3) that has been proposed to contain one or more tumor suppressor genes. We report here consistent downregulation of AHRR mRNA in human malignant tissue from different anatomical origins, including colon, breast, lung, stomach, cervix, and ovary, and demonstrate DNA hypermethylation as the regulatory mechanism of AHRR gene silencing. Knockdown of AHRR gene expression in a human lung cancer cell line using siRNA significantly enhanced in vitro anchorage-dependent and -independent cell growth as well as cell growth after transplantation into immunocompromised mice. In addition, knockdown of AHRR in non-clonable normal human mammary epithelial cells enabled them to grow in an anchorage-independent manner. Further, downregulation of AHRR expression in the human lung cancer cell line conferred resistance to apoptotic signals and enhanced motility and invasion in vitro and angiogenic potential in vivo. Ectopic expression of AHRR in tumor cells resulted in diminished anchorage-dependent and -independent cell growth and reduced angiogenic potential. These results therefore demonstrate that AHRR is a putative new tumor suppressor gene in multiple types of human cancers.

Introduction

The aryl hydrocarbon receptor repressor (AHR, aryl hydrocarbon receptor; AHRR, AHR repressor; ARNT, AHR nuclear translocator; bHLH, basic helix-loop-helix; FAK, focal adhesion kinase; LOH, loss of heterozygosity; NCI, National Cancer Institute; RPMI-10, RPMI supplemented with 10% FBS; XRE, xenobiotic response element.) is a newly discovered member of the growing superfamily of basic helix-loop-helix/Per-ARNT-Sim (bHLH/Per-ARNT-Sim) transcription factors, which includes the aryl hydrocarbon receptor (AHR) (1) and hypoxia inducible factor 1 (HIF1) (2), among others. AHR represses the transcription activity of AHR by competing with this transcription factor for heterodimer formation with the AHR nuclear translocator (ARNT) (3) and subsequently binding to the xenobiotic response element (XRE) sequence (4), functioning as a naturally occurring repressor; ARNT, AHR nuclear translocator; bHLH, basic helix-loop-helix; FAK, focal adhesion kinase; LOH, loss of heterozygosity; NCI, National Cancer Institute; RPMI-10, RPMI supplemented with 10% FBS; XRE, xenobiotic response element.

Conflict of interest: The authors have declared that no conflict of interest exists.


Nonstandard abbreviations used: AHR, aryl hydrocarbon receptor; AHRR, AHR repressor; ARNT, AHR nuclear translocator; bHLH, basic helix-loop-helix; FAK, focal adhesion kinase; LOH, loss of heterozygosity; NCI, National Cancer Institute; RPMI-10, RPMI supplemented with 10% FBS; XRE, xenobiotic response element.

Results

AHRR is downregulated in tumor cells. Our results show a consistent downregulation of AHRR throughout all the tumor types assessed in this study, including colon, breast, lung, stomach, cervical, and ovarian, when compared with normal tissues of the same anatomical origin (Figure 1). In preliminary experiments lung, breast, stomach, and colon primary tumors showed substantial downregulation...
tion of AHRR when compared with normal tissue from the same patient, adjacent to the tumor (Figure 1A). Interestingly, some of the hyperplastic or adenomatous nonmalignant colon polyps showed a smaller (up to 40%) downregulation of AHRR (Figure 1B), suggesting a direct relationship between levels of AHRR expression and degree of transformation in colon cancer. In a separate experi-
was found in seminoma testicular tumors (it has been reported that seminomatous germ cell tumors rarely exhibit promoter hypermethylation; ref. 22). Interestingly, levels of promoter hypermethylation were closely correlated with cancer grade in cervical and esophageal specimens: low-grade precancerous lesions showed the lowest levels of promoter methylation (4.2% and 0%, respectively), higher levels were observed in high-grade lesions (41% and 28%, respectively), and virtually all established cervical tumors (95.5%) and 52% of esophageal tumors were methylated. Further supporting evidence that hypermethylation is a main regulatory mechanism of AHRR silencing in tumors comes from experiments in which tumor cells are exposed to demethylating agents. Treatment of tumor cells with 5-aza-dC and/or trichostatin A significantly reactivated AHRR expression (more than 1.5-fold increase) in 71.5% of the tumor cell lines included in the experiment (Supplemental Table 3). These data thus show that epigenetic alterations (i.e., hypermethylation) are largely responsible for down-regulation of AHRR in cancer cells. Analysis of the 5′ regulatory region of AHRR showed several binding motif sites were found in the same region affected by hypermethylation including AP-2, c–Est-1, and Elk-1 (Supplemental Table 4). Especially interesting was a short sequence, located close to the start codon (–55 to –41 bp from the start codon), which contained potential binding sites for several members of the bHLH family of transcription factors including AHR and HIF.

The collective data shown above strongly suggest that AHRR may function as a tumor suppressor gene in cancers from different tissue origins. It is assumed that the loss of tumor suppressor gene function correlates with an aggressive tumorigenic phenotype (23). siRNA technology in combination with in vitro cell-based growth assays (such as anchorage-independent growth assay) has been recently recognized as a successful strategy for identification of new tumor suppressor genes (24). In this context, and to better understand the functional relevance of AHRR as a tumor suppressor gene, we artificially suppressed its expression by siRNA technology in the lung tumor A549 and normal breast MCF10A cell lines (Supplemental Figure 3). Transfected cells were tested for a number of established hallmark traits of the tumorigenic phenotype such as growth and colony formation both in vivo and in vitro resistance to apoptosis, migration, and angiogenic potential (25).

Silencing of AHRR enhances tumor growth in vitro and in vivo through deregulation in cell cycle control. The hallmark of all neoplastic development is deregulated cell proliferation (25). Downregulation of AHRR resulted in enhanced cell growth potential (Figure 2A). After 5 days of transfection, a difference of 4.3- and 2.6-fold in growth was observed between A549E and A549G/F, respectively. Cell cycle analysis on these cells showed that the cell number on G1/M phase of A549F/G was significantly higher than that of A549E (Figure 2, B–D). Consistently, G2/M phase was significantly decreased in these cells. In addition, we determined that several prooncogenic factors were significantly elevated on A549G/F, including Ras, PKCa, and cyclin A, while tumor suppressor factors such as PTEN and Ku80 were downregulated (Table 2). Further strengthening these results, downregulation of AHRR enhanced the ability of A549F and A549G to form colonies on anchorage-independent clono-

### Table 1

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>No. studied</th>
<th>Methylated (%)</th>
<th>UM/M (%)</th>
<th>M/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal testis</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Testicular cancer</td>
<td>79</td>
<td>44.3</td>
<td>42.8</td>
<td>57.2</td>
</tr>
<tr>
<td>Seminoma</td>
<td>15</td>
<td>13.3</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Nonseminoma</td>
<td>64</td>
<td>51.6</td>
<td>42.4</td>
<td>57.6</td>
</tr>
<tr>
<td>Normal cervix</td>
<td>44</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cervical cancer</td>
<td>66</td>
<td>95.5</td>
<td>81</td>
<td>19</td>
</tr>
<tr>
<td>Cell lines</td>
<td>9</td>
<td>100</td>
<td>11.1</td>
<td>88.9</td>
</tr>
<tr>
<td>Primary tumors</td>
<td>57</td>
<td>94.7</td>
<td>92.6</td>
<td>7.4</td>
</tr>
<tr>
<td>Precancer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-grade</td>
<td>48</td>
<td>4.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>High-grade</td>
<td>39</td>
<td>41</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Normal ovary</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>14</td>
<td>100</td>
<td>21.4</td>
<td>78.6</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>6</td>
<td>67</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Colon cancer</td>
<td>8</td>
<td>100</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Normal esophagus</td>
<td>8</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Low-grade dysplasia</td>
<td>8</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>High-grade dysplasia</td>
<td>7</td>
<td>28</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Tumor</td>
<td>23</td>
<td>52</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

M/M, only methylated allele present; UM/M, unmethylated and methylated alleles present; ND, not done.

AHRR satisfies Knudson’s "two-hit" hypothesis. Because of the downregulated expression of the AHRR gene in tumors from different tissue origins, and the frequent LOH at the 5p15 chromosomal region (12), we reasoned that AHRR might be a potential tumor suppressor gene. Knudson’s “two hits” hypothesis considers tumor suppressor genes by their functional inactivation achieved by a combination of LOH, hypermethylation of the gene’s promoter, and mutations in the gene’s coding region (20). Although, as expected from previous studies (12, 21), a number of polymorphisms and evidence for frequent LOH of this region was obtained in this study (Supplemental Table 2), no evidence of pathogenic mutations in the coding region of the AHRR gene was found, pointing to hypermethylation as a possible mechanism responsible for its steady downregulation in tumors. Promoter hypermethylation was found in over 78% of the tumors examined (Table 1 and Supplemental Figure 2), while no methylation was found in normal tissue. Remarkably, almost all ovarian, cervical, and colon tumors exhibited promoter hypermethylation. The lowest hypermethylation rate (13%) was found in seminoma testicular tumors (it has been reported...
Figure 2
Effects of siRNA-induced silencing of AHRR on growth. (A) On a time-course MTT assay, A549G (squares) grew 4.3-fold and A549F (triangles) grew 2.6-fold faster than the empty nonsilenced A549 cells (circles, A549E; diamonds, A549SR). No differences were observed between A549E and A549SR. (B–D) Cells deficient in AHRR (A549F [C] and A549G [D]) showed a significant shift in the number of cells towards S and G2/M phases as compared with the control A549E (B). Consistently, a reduction in the number of cells in G0/G1 phase was observed in A549F/G. (E and F) Artificial downregulation of AHRR enhanced A549 (E) and MCF10A (F) colony formation (60% and 300% increase in average colonies for A549F/G and A549E, respectively). MCF10A-F and MCF10A-G were able to clone in soft agar while, as expected, MCF10A-E did not form colonies. All clonogenic assays were run in triplicate. (G and H) Comparison of the morphology of the colonies formed by A549E (G) and A549G (H). A549E grew in compact spheroids with defined contours. A549G colonies showed irregular morphology and cells detached and partially separated from the core of the colony. Original magnification, ×20. (I) In an in vivo experiment, A549 cells transfected with a siRNA for AHRR showed a significant increase in xenograft tumor volume 8 weeks after injection when compared with empty plasmid–transfected A549 cells (3-fold increase for A549F [squares] compared with A549E [triangles] and 7-fold increase for A549G [diamonds] compared with A549E). n = 10 animals/group. *P < 0.05; **P < 0.01; ***P < 0.001.
Table 2: Comparison of the protein expression levels between A549G and A549E

<table>
<thead>
<tr>
<th>Protein name</th>
<th>SwissProt ID</th>
<th>Fold change</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vimentin</td>
<td>P08670</td>
<td>+33.07</td>
<td>Overexpression is associated with metastatic potential</td>
</tr>
<tr>
<td>HES-1</td>
<td>NF</td>
<td>+6.67</td>
<td>Regulates Notch and potentially plays a role in tumorigenesis</td>
</tr>
<tr>
<td>LAP2</td>
<td>O62733</td>
<td>+6.13</td>
<td>May play an important role in nuclear envelope reassembly at the end of mitosis and/or anchoring of the nuclear lamina and interphase chromosomes to the nuclear envelope</td>
</tr>
<tr>
<td>TA01</td>
<td>O88664</td>
<td>+4.66</td>
<td>Regulates MAPK pathway</td>
</tr>
<tr>
<td>Casein kinase II a/a</td>
<td>P19139</td>
<td>+4.17</td>
<td>Related with transformed phenotype and survival of cancer cells</td>
</tr>
<tr>
<td>Stat6</td>
<td>P42226</td>
<td>+3.36</td>
<td>Mediates repression of immunosurveillance</td>
</tr>
<tr>
<td>Phosphospecific FAK</td>
<td>Q05397</td>
<td>+3.13</td>
<td>Enhances cell motility, invasion, and tumor progression</td>
</tr>
<tr>
<td>PKCα</td>
<td>P17252</td>
<td>+1.8</td>
<td>Implicated in events leading to keratinocyte differentiation, epidermal tumor promotion, and cutaneous inflammation</td>
</tr>
<tr>
<td>Cyclin A</td>
<td>P20248</td>
<td>+1.5</td>
<td>Key cell cycle regulator, the expression of which is found to be elevated in a variety of tumors</td>
</tr>
<tr>
<td>PTEN</td>
<td>Q00633</td>
<td>−1.8</td>
<td>Tumor suppressor gene, the downregulation of which enhances migration</td>
</tr>
<tr>
<td>Stat1</td>
<td>A46159</td>
<td>−1.9</td>
<td>Mediates growth inhibitory signals and contributes to the host rejection of tumors</td>
</tr>
<tr>
<td>MCM5</td>
<td>P33992</td>
<td>−2.4</td>
<td>Enhances Stat1 transactivation function</td>
</tr>
<tr>
<td>Ku80</td>
<td>P13010</td>
<td>−2.7</td>
<td>Mediates inhibition of hepatocellular carcinoma development</td>
</tr>
<tr>
<td>PKB</td>
<td>P31749</td>
<td>−2.96</td>
<td>Leads to downregulation of tumor suppressor gene p53</td>
</tr>
<tr>
<td>DAP kinase</td>
<td>P53355</td>
<td>−3.65</td>
<td>Loss of expression links suppression of apoptosis to metastasis</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>P12830</td>
<td>−4.65</td>
<td>Loss of expression represents a key step in the acquisition of the invasive phenotype for many tumors</td>
</tr>
<tr>
<td>Bcl-x</td>
<td>O00633</td>
<td>−1.8</td>
<td>Potent antiapoptotic factor</td>
</tr>
<tr>
<td>Bip/GRP78</td>
<td>P11021</td>
<td>BL</td>
<td>Highly induced in growing tumors</td>
</tr>
<tr>
<td>Ras</td>
<td>P01112</td>
<td>BL</td>
<td>Oncogene that promotes cell progression</td>
</tr>
</tbody>
</table>

BL, below the detection limit in A549 and present in A549G; NF, not found in SwissProt database. Only data generated from good-quality signals with fold changes >1.5 were considered.

Of note, the enhanced clonogenic capability was accompanied by differences in the morphology of the colonies. A549E typically grew in colonies displaying similar morphology to the one observed in A549F/G. In contrast, A549F/G were able to grow in colonies displaying similar morphology to the one observed in A549E. Overall, the data described above clearly demonstrate a link between AHRR downregulation and tumor cell growth in vitro. To further address this finding in an in vivo model of tumorigenesis, we injected A549E/F/G cells into nude mice and followed the development of xenograft tumors over time. A549F- and A549G- induced tumors showed a 3- and 7-fold increase in volume, when compared with tumors generated by A549E (Figure 2E). Smaller differences were found between A549E and A549F (3-fold increase). These data further suggest that downregulation of AHRR is a contributing factor to enhanced tumorigenicity.

AHRR silencing protects against apoptosis and enhances angiogenic potential, migration, and invasion in tumor cells. In addition to growth deregulation and clonal expansion, resistance to apoptosis (27), enhanced angiogenic potential (28), and motility (25) lie at the heart of all tumor development. Here we show that silencing of AHRR confers resistance to apoptosis induced by overnight exposure to various proapoptotic signals (Figure 3A). Proteomics expression profile of A549G cells showed a significant elevation of the antiapoptotic factor Bcl-x (Table 2), suggesting potential explanatory mechanisms. We also studied the angiogenic potential of transfected A549 cells in vivo using DIVAA. An 8-fold increase in neovascularization-related fluorescence was observed in A549G when compared with A549E (Figure 3B). Similarly, A549F exhibited higher angiogenic potential (5-fold increase) compared with A549E. Although sprouting of capillaries from quiescent vasculature on the tumor is a prerequisite for metastasis (28), enhanced motility and invasiveness are also required. The morphological alterations observed in A549F/G in clonogenic assays (Figure 2, F and G) suggest loss of cell-cell contact within the colonies and possibly higher migratory potential. Additionally, A549F/G showed increased (7- and 4.5-fold increase, respectively) migratory potential over A549E (Figure 3C). Similar results were obtained when testing the invasive potential of A549-transfected cells. Silencing of AHRR correlated with increased invasion, as observed through Matrigel staining (Figure 3C). These results are consistent with upregulation of prometastatic factors such as vimentin or phosphorylated focal adhesion kinase (FAK) and loss of E-cadherin, which is associated with loss of cell-cell interactions and enhanced invasive phenotype, in cells with artificially silenced AHRR (Table 2).

Ectopic expression of AHRR opposes the effect of siRNA for AHRR. Overexpression of AHRR was achieved in a transient fashion in A549 lung adenocarcinoma cells (Supplemental Figure 3C). AHRR was initially described as a repressor of the AHR, and our hypothesis contemplates a suppressor activity of AHRR linked to the AHR tumorigenic potential. To confirm biological activity of the overexpressed AHRR, we measured levels of the AHR-induced cancer-related gene CYP1A1. As expected, transfection of AHR-induced CYP1A1 expression and cotransfection of AHRR counteracted the effect of AHR on reducing the levels of CYP1A1.
4A), therefore confirming biological activity of the overexpressed AHRR as a repressor of AHR. Interestingly, A549-AHRR showed reduced growth rate over 3 days compared with A549-empty, as assessed by Electric Cell-Substrate Impedance Sensing (Figure 4B). Consistently, AHRR transfer resulted in inhibition of anchorage-independent growth in clonogenic assays (Figure 4C). Colonies were morphologically similar and reached the same size (Figure 4B), although significantly fewer colonies were found in A549-AHRR samples. Also, using a modified tube formation assay in which PAE-GFP were cocultured (in separate Matrigel compartments) with transfected A549 cells (Figure 4D), we demonstrated reduced tube formation after exposure to A549-AHRR compared with cells exposed to A549-empty, showing that overexpression of AHRR reduced the angiogenic potential of A549.

Discussion

Tumor formation arises as a consequence of the acquisition of unique cellular capabilities, which involve, among others, deregulation of cell proliferation, resistance to apoptosis, enhanced cell motility, augmented angiogenic potential, and anomalies in cell-cell interaction and the microenvironment, resulting in invasion and metastasis (25). Over the last several years, we have learned that this process is regulated by a relatively small subset of genes that act by either enhancing (oncogenes) or diminishing (tumor suppressor genes) the final malignant outcome (29). Finding tumor suppressor genes is challenging, and although approaches based on analysis of LOH have been proven to be successful (29), we now know that multiple lines of evidence, including functional implications, are needed to confirm the identification of newly defined tumor suppressor genes (30). Here we provide clinical, genetic, and functional evidence supporting the hypothesis that silencing of the AHRR occurs in tumors from different tissue origins and that this mute expression results in an aggressive tumorigenic phenotype, thus suggesting that AHRR plays an important role in suppressing tumor formation in humans.

In this study we demonstrate downregulation of AHRR throughout all the tumor types assessed, including colon, breast, lung, stomach, cervical, and ovarian, when compared with normal tissues of the same origin. Functional inactivation of tumor suppressor genes is achieved by a combination of LOH, hypermethylation of the gene’s promoter, and mutations (20, 31). We have found high rates of LOH (together with several new polymorphisms) and hypermethylation in the promoter region of the AHRR gene in cervical, testicular, and ovarian tumors. The majority of tumors, particularly ovarian cancer and testicular germ cell tumors, exhibited only methylated alleles, which can result from LOH of one allele and methylation of the other or methylation of both alleles. The first mechanism may certainly account for a proportion of cases, since 5p15 LOH is a common event in cervical cancer and testicular germ cell tumors (12, 21, 32–34). These data, together with the reactivation of AHRR expression after exposure to demethylating agents, are consistent with Knudson’s 2-hit hypothesis (20, 31) and indicate that LOH and hypermethylation constitute the main mechanism for the silencing of AHRR in tumors. Inactivation of tumor suppressor genes can represent an early event and a prerequisite for clonal expansion. In support of this idea we, have found...
a direct relationship between hypermethylation of the AHRR promoter region and tumor grade in cervical and esophageal cancer. Further supporting this idea, a small set of colon polyps (which we can consider as a naturally occurring example of premalignant lesions) showed intermediate downregulation of AHRR compared with colon tissues from healthy patients and frank colon tumors. This leads us to postulate that a reduced dosage of AHRR in the polyps, rather than its absolute absence, may contribute to cancer susceptibility in initial stages of the carcinogenic process.

Hypermethylation in the neighborhood of the start codon has been shown to suppress gene expression in tumor cells by either interfering with RNA polymerase II initiation or transcription factor binding (35). This region is therefore more likely to contain binding sequences for transcription factors relevant in AHRR regulation in tumor cells. Several regions of the promoter of the AHRR gene have been shown to be potentially relevant for the regulation of its expression (36, 37). We have found that the sequence contained between –55 bp and –41 bp from the start codon of the AHRR gene (which we have shown to be hypermethylated in tumors) is rich in potential binding sites for several members of the bHLH family of transcription factors, some of which are known to be upregulated in tumor cells (AHR, c-myc/Max, and HIF-1). Upregulation of these transcription factors in cancer cells would potentially correlate with an increase in the levels of AHRR and an enhanced cancer suppressive action. However, hypermethylation of this promoter region and LOH prevent AHRR expression with
concomitant loss of its repressor function and enhancement of the tumorigenic phenotype (Figure 5). Due to the significant length of the regulatory region on the AHR gene, additional studies targeting this area are guaranteed to deepen our understanding of the role that methylation plays on AHR expression in tumor cells.

Functional evidence of the tumor suppressive nature of AHR arises from the study of the malignant phenotype in cells with artificially altered AHR expression. Independent of the etiology of different tumors, deregulated cell proliferation together with suppressed apoptosis constitute a common platform upon which all neoplastic evolution occurs. Here we demonstrate that downregulation of AHR enhances growth potential through deregulation in cell cycle control and resistance to apoptosis (potentially through deregulation of cyclin A and Bcl-x, respectively). AHR inactivation is also linked to enhancement in anchorage-independent growth of tumor cells. Artificial upregulation of AHR results in impaired anchorage-dependent and -independent growth. In agreement with these data, a recent report shows that overexpression of AHR results in blockage of growth in the breast cancer cell line MCF-7 (18). Interestingly, partial silencing of AHR in nontumoral MCF10A (which lack the ability of anchorage-independent growth; ref. 26) enables them to clone in soft agar, further supporting the important role of AHR as a negative regulator of clonogenicity. Recently it has been shown that immortalized mouse mammary fibroblasts lacking AHR have impaired tumorigenicity in a subcutaneous mouse xenograft model (38) and that its overexpression promotes progression through the cell cycle (39), triggering a malignant phenotype in vivo (8, 9). Tumors cells showing inactivation of the AHR negative regulator AHRR showed enhanced tumorigenic potential, while AHR activation resulted in diminished tumorigenic potential, in agreement with the logical hypothesis that inactivation of AHRR should lead to similar physiological consequences. The proteomic profile of cells silenced for AHR shows that several prooncogenic factors such as Ras, HES-1, and casein kinase Ila and anti-apoptotic factors such as Bcl-x are elevated, while tumor suppressor molecules such as PTEN are diminished. This could explain the enhanced growth and apoptosis resistance in these cells. These collective data implicate AHR as an important regulator of the overall tumor development, the expression of which inversely correlated with cell growth and protected against apoptosis in cancer cells.

One of the essential capabilities that enhances cancer cells’ tumorigenicity is their ability to migrate and become invasive. Here we show that silencing of AHR tracks with increased motility and invasive potential in tumor cells and is accompanied by loss of E-cadherin, augmentation in vimentin, and phosphorylated FAK expression. Deregulation of these proteins has been previously related to an increase in migratory potential and a more invasive phenotype in tumors (40–42). Consistent with our results, a recent report has shown that cells lacking AHR expression show downregulation of FAK and impaired migratory capabilities (38). The increased motility and the defined proteomic profile in AHRR-deficient cells could also account for the abnormal colony morphology observed in clonogenic assays in which cells appeared to detach and migrate away from the main core of the colony. Tumor growth and metastasis rely on angiogenesis, the induction of new sprouting capillaries from quiescent vasculature (28). AHR expression in tumor cells inversely correlates with their angiogenic potential. Tumor cells ectopically overexpressing AHR showed lower angiogenic potential, while tumor cells in which AHR expression was blocked showed enhanced angiogenic potential. In summary, our data support that enhanced growth, clonal expansion, motility, and angiogenic potential, together with increased resistance to apoptotic signals, are cellular capabilities gained as a result of downregulation of AHR expression and account for the xenograft growth potential of AHRR low-expressing cells in vivo.

The clinical, genetic, and functional data presented here implicate AHR as a key regulator in the tumorigenic process whose down-

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**Figure 5**

Working model of the tumor suppression mechanism for AHRR integrated in a gene regulation feedback loop with AHR. (A) In normal cells, activation of AHR causes induction of AHRR through XREs present in its promoter. AHRR acts as a negative regulator of AHR by competing for binding to ARNT and XREs present in the promoter regulatory region of variety of genes. The balanced relationship between the positive and negative transactivation signals of AHR and AHRR results in cellular homeostasis. (B) In cancer cells, methylation of the AHRR promoter and LOH cause blockade of its expression despite the higher levels of AHR. Thus absence of AHRR eliminates competition for binding to ARNT and XREs, which results in an imbalance between positive and negative transactivation signals, thereby causing the induction of a battery of genes related to tumorigenesis and cancer progression.

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regulation (a consequence of methylation in tumor cells) induces pleiotropic effects, leading to tumor promotion and progression. Our data are consistent with the hypothesis that AHRR acts as a tumor suppressor gene in cancers from different tissue origins. The broad spectrum of signaling pathways regulated by AHRR renders this molecule an attractive biological target for developing new therapeutic intervention approaches against human cancers.

Methods

Normal tissue, tumor specimens, and cell lines. Colon specimens (normal controls and polyps) were obtained from standard colon pinch biopsies obtained during routine colonoscopy of patients without known predisposition to colon cancer and from patients with hereditary nonpolyposis colorectal cancer under protocols approved by the Internal Review Boards of both the National Cancer Institute (NCI) and the National Naval Medical Center. Lung cancer and distal normal tissue was obtained from the University of Colorado Cancer Center Lung SPORE Tissue Bank. Tumors and corresponding normal tissue were snap frozen and stored in liquid nitrogen. Total RNA from pairs of individual human tumor and adjacent normal tissues (colon, lung, stomach, and breast) were obtained from Ambion. DNA isolated from 10 normal peripheral blood cells and a total of 66 tumors from cervix uteri (9 cell lines and 57 primary tumors) were utilized as described previously (43). All primary cervical cancers were squamous cell carcinoma of stages Ib to IV. Cervical cancer cell lines (HT-3, ME-180, HeLa, M5751, C-4I, C-33A, CaSi, SW756, and SiHa) were obtained from the ATCC. DNA obtained from 4 normal testes and 79 testicular germ cell tumors were used as previously described (44). All tumors (at least 60% tumor cellularity) were obtained after appropriate informed consent and approval of the protocols were obtained from the NIH IACUC. Total RNA from normal cervixes was purchased from 3 different sources (Ambion, Stratagene, and BioChain). DNA isolated from 2 normal ovarian cell lines (immortalized ovarian surface epithelium – IOSE 80 and IOSE 120; ref. 45) and 14 ovarian carcinoma cell lines (A2780, AD10, OVCA429, A222, C70, OVT[2](OF), CAO3, 222, A547, A364, OVCA3, UC1I01, SKOV3, and UC1I07) were also included in this study (46). All the ovarian cell lines were cultured as previously described (47). The bronchoalveolar carcinoma cell line A549, small lung cancer H417, and carcinoid H720 were obtained from ATCC. MCF10A was kindly provided by David Salomon (NCI) and maintained as previously described (26). The porcine endothelial cell PAE was obtained from Lena Claesson-Welsh (The Rudbeck Laboratory, Uppsala University, Uppsala, Sweden) through MTA agreement and subcultured as previously described (48).

RNA reverse transcription and real-time PCR. Reverse transcription was performed using the SuperScript First-Strand Synthesis System (Invitrogen). PCR was performed using the ABI Prism 7900 Sequence Detection System (Applied Biosystems). Amplification was performed using 10 μM each of the following sense and antisense primers: AHRR sense, 5’-CTTATG-GCCTTGTCTGGTGCG-3’; AHRR antisense, 5’-TGTTCACTACAGCTCGCAGTATGGGA-3’; 18S rRNA sense, 5’-ATGTCTTCTGATCGTATGTCGGCG-3’; 18S rRNA antisense, 5’-ATTCTAGCTGCGGTATCCAGG-3’ and SYBR Green Master Mix (Applied Biosystems).

DNA. The standard amplification program was according to the following cycle scheme: initial denaturation of the samples at 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 60 s. Fluorescence was measured in every cycle, and a defined single peak in the melting curve was obtained for all amplicons, thus confirming the specificity of the amplification. The final mRNA levels of the genes studied were normalized according to the 18S rRNA concentration of each sample.

Mutational analysis. Primer sets covering coding exons 2–11, and 5’ of exon 11 of AHRR gene were designed to amplify genomic DNA up to 240 bp (Supplemental Table 1). Single-strand conformation polymorphism (SSCP) analysis was performed on PCR products generated from 30 cervical cancers in the presence of [32P]dCTP and running 6% non-denaturing polyacrylamide gels containing 10% glycerol. Purified PCR products were sequenced to identify mutations.

Promoter methylation analysis by methylation-specific PCR. A CpG island (1,222 bp) spanning intron 2/intron 2 of the AHRR gene was identified using GraiExp Grai Experimental Gene Discovery Suite (http://grai. lsd.onl.nl/grailexp/). Two sets of primers for amplification of methylated and unmethylated DNA were designed by standard methods and were as follows: AHRR-M-F3, 5’-GTACCTGATTTCTTCTGCGG-3’; AHRR-M-R3, 5’-CCGATAAATCTGCATACGCA-3’; AHRR-U-F3, 5’-GTATGTTATTTTTTGGTGG-3’; AHRR-U-R3, 5’-CCCCATAACTCCTCAATACA-3’ (–208 to 27 bp); and AHRR-M-F2, 5’-TTCGCTGTTTCCGTGTTTGTGC-3’; AHRR-M-R2, 5’-CCAACGACCGGAAACTAAACTC-3’; AHRR-U-F2, 5’-GGTTGGTGTGTTGAGTTTT-3’; AHRR-U-R2, 5’-AAAACCTACATCCATACCCCTCA-3’ (–333 to –178 bp). Genomic DNA was treated with sodium bisulfite as previously described (43). Placental DNA treated in vitro with SssI methyltransferase (New England Biolabs) and normal lymphocyte DNA treated similarly with sodium bisulfite were used as controls for methylated and unmethylated templates, respectively. PCR products obtained from standard reaction were run on 2% agarose gels and visualized after ethidium bromide staining.

5-Aza-2’-deoxycytidine and trichostatin treatment and analysis of gene expression. Tumor cell lines (cervical: C-4I, CaSi, C-33A, HT-3, siHa, SW756, MS751m, ME-180, HeLa; testicular: CL2102EP, 2CL-21218A; and lung: A549, H720, H417) were treated with demethylating agent 5-Aza-2’-deoxycytidine (Sigma-Aldrich) for 5 days at a concentration of 5 μM, HDAC-inhibiting agent trichostatin A (Sigma-Aldrich) at a final concentration of 250 nM for the last 24 hours, or a combination of both. Total RNA isolated from treated and untreated cell lines and the total RNA and polyA+ RNA from normal cervix obtained from commercial sources were reverse transcribed using random primers and the ProSTAR first-strand RT-PCR kit (Stratagene).

Analysis of transcription factor binding sites in the promoter region of the AHRR gene. The 155-bp region corresponding to –333 bp to 27 bp from the start codon of the AHRR gene was searched for transcription factor binding sites using MatInspector software (49) (Genomatix Software GmbH). Only sequences with core similarity of 1, matrix similarity greater than 0.9, and optimized matrix threshold greater than 0.8 were considered.

Construction of expression plasmids and generation of transfectants. The mammalian expression vector pSuper.neo (OligoEngine) was used for expression of an AHRR-specific siRNA in A549 and MCF10A cells. Two gene-specific inserts (5’-AGAGCTTCTCCAAAGTCTGG-3’ and 5’-GGCTGCTGTGGGAGTTCTTT-3’) were cloned into the pSuper.neo backbone, and the final plasmids were referred to as pSuper.neo-F and pSuper.neo-G, respectively. A control vector containing no insert (empty vector, referred to as pSuper.neo-E) or a plasmid containing a scramble sequence (referred to as pSuper.neo-sr) or wild-type untransfected cells served as nonsilencing controls. To assess the appropriateness of the nonsilencing controls, equal levels of AHRR mRNA were confirmed in A549 transfected with pSuper.neo-sr (A549SR), untransfected cells (A549WT), and A549 transfected with the empty vector (A549E) (Supplemental Figure 1A). Similar growth and migratory rates among A549E, A549SR, and A549WT were also observed (Supplemental Figure 1B and C). The complete open reading frame of the human AHRR gene was amplified by PCR, cloned into the pcDNA 3.1 TOPO TA mammalian expression vector (Invitrogen), and transiently transfected into A549 cells (A549-AHRR). Control vector (pcDNA3.1) containing no insert was used to generate negative control cells (A549-empty). The open reading frame of GFP was cloned into the pcDNA3.1 TOPO TA
backbone (pcDNA3.1-GFP). All vectors were sequenced in the forward and reverse directions to verify the insertion of the AHRR and GFP sequences and the lack of insert in the control plasmids. A549 and MCF10A cells were transfected with plasmids pSuper.neo-E, pSuper.neo-F, or pSuper.neo-G using FuGENE6 (Invitrogen) and exposed to the appropriate media containing 800 μg/ml geneticin (Invitrogen). A549 was transiently transfected with pAHRR and pcDNA3.1 using the AMAXA nucleofector system. Expression of AHRR in both siRNA stable transfecants and transient over-expressors was assessed by real-time PCR (Supplemental Figure 3). Pae cells were transiently transfected with pcDNA3.1-GFP (PAE-GFP). Selection of expressing cells was achieved by flow cytometry cell sorting using FACScan and CellQuest software (Becton Dickinson).

Growth assays. Proliferation of cells stably transfected with siRNA for AHRR or the empty vector was assessed by MTT assay. The dye and solubilization solutions (Promega Proliferation Assay) were added every day for 5 days to separate 96-well plates, and absorbance was measured at 570 nm with a Spectra Rainbow (Tecan) plate reader. Growth potential of A549-AHRR was assessed by Electric Cell-Substrate Impedance Sensing (ECIS Model 9600; Applied Biophysics) (50). A549-AHRR and A549-empty cells were seeded in 8W10E+ plates in RPMI supplemented with 10% FBS (RPMI-10). Measurements were performed for 4 days at 15 kHz.

Cell cycle analysis. For DNA content analysis, 3 × 10⁶ cells (A549E, A549F, and A549G) were fixed, permeabilized in 70% ethanol, and washed twice in PBS (pH 7.4). Cells were then resuspended in 1 ml of PBS containing 50 μg propidium iodide (Sigma-Aldrich) and 1,000 units of DNase-free RNase (Sigma-Aldrich) and incubated for 30 min at 37°C. The samples were run on a FACScan using CellQuest software and analyzed using the Sync Wizard option of MODFIT LT 2.0 (Verity Software House Inc.).

Soft agar clonogenic assay. The anchorage-independent growth of A549E, A549F, A549G, A549-empty, and A549-AHRR cells was examined by soft agar clonogenic assay. Briefly, 5,000 cells were resuspended in 1.5 ml of the culture media containing 10% FBS and 0.3% agarose and plated in 6-well plates with 1.5 ml of pre-solidified culture media in 0.5% agar containing 10% FBS. Plates were incubated at 37°C for 4 weeks, and colonies larger than 0.1 mm in diameter were counted. The same procedure was followed for MCF10A-E, MCF10A-F, and MCF10A-G seeding 20,000 cells per well.

Xenografts. A549 cells transfected with the AHRR siRNA plasmids or the empty vector were injected into the flanks of athymic (nude) mice (1 × 10⁶ cells/mouse). The mice (10 animals/cell line) were checked daily for tumor formation by palpation, and tumor volume was estimated by measuring its size in 3 dimensions twice a week. This experiment was conducted in a blind fashion under animal protocol approved by the Animal Care and Usage Committee of NCI–Frederick Cancer Research Center.

Apoptosis assay. Cells were suspended in RPMI-10, seeded in 96-well plates, and incubated overnight. Then the plates were washed with RPMI media and exposed to: serum-free RPMI, RPMI-10 and 0.5 μM of MK886 (Sigma-Aldrich). After overnight incubation, early apoptosis was determined as the production of caspase-3/7 products using the Apo-ONE Homogenous Caspase-3/7 Assay (Promega).

Migration assays. Chemotaxis was assayed in 8-μm pore, 96-well ChemoTx plates (Neuroprobe). Cells (5 × 10⁴) were placed in the upper chambers and the lower wells were filled with RPMI-10. After a 4-hr migration period at 37°C, nonmigrating cells were wiped off the top surface of the membrane. Then the membranes were fixed and stained with Hema3 (Biochemical Sciences Inc.), and the cells trapped in the pores of the membrane were counted. Eight repeats were performed per sample.

Tube formation assays. The same number of A549-empty or A549-AHRR cells were seeded in 96-well plates. After the cells completely attached, the plates were washed 3 times with serum-free RPMI. Then 50 μl of base membrane extract (Trevigen) was laid down in each well. PAE-GFP cells (15,000) were then added on top of the gelled BME, and images were acquired with a fluorescent microscope after 3.5 hours.

Directed in vivo angiogenesis assay. Quantitation of angiogenesis was done using directed in vivo angiogenesis assay (DIVAA) as previously described (51). Briefly, 10-mm-long surgical grade silicone tubes with only 1 open end (angioreactors) were filled with 20 μl of Matrigel alone or Matrigel containing 10,000 cells. After the Matrigel solidified, the angioreactors were implanted into the dorsal flanks of anesthetized athymic nude mice (NCl colony). After 11 days, the mice were injected i.v. with 25 mg/ml FITC-dextran (100 μl/mouse; Sigma-Aldrich) 10 min before removing angioreactors. Quantitation of neovascularization in the angioreactors was determined as the amount of fluorescence trapped in the implants and was measured in a SpectraFluor microplate reader (Tecan). This protocol was approved by the NIH IACUC.

Proteomic analysis. A549G and A549E cells (10⁶ cells per assay) were suspended in boiling lysis buffer (10 mM Tris–HCl [pH 7.4], 1 mM sodium orthovandate, 1% SDS) and homogenized by sonication. The protein content of each lysate was quantified by the bicinechonic acid assay (Pierce Biotechnology) and subsequently processed by the PowerBlot facility (Becton Dickinson) to measure the expression level of 280 different signal-transducing proteins related to cell cycle regulation and proteins susceptible of phosphorylation. The experiment was performed in triplicate.

Statistics. Comparisons between groups were made using the 2-tailed Student’s t test and the Mann-Whitney U test. Plotted data were expressed as mean ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001 are significance values used in the figures.

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