The Septin-Binding Protein Anillin Is Overexpressed in Diverse Human Tumors

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Abstract

Anillin is an actin-binding protein that can bind septins and is a component of the cytokinetic ring. We assessed the anillin expression in 7,579 human tissue samples and cell lines by DNA microarray analysis. Anillin is expressed ubiquitously but with variable levels of expression, being highest in the central nervous system. The median level of anillin mRNA expression was higher in tumors than normal tissues (median fold increase 2.58; 95% confidence intervals, 2.19-5.68, P < 0.0001) except in the central nervous system where anillin mRNA levels were lower in tumors. We developed a sensitive reverse transcription-PCR strategy to show that anillin mRNA is expressed in cell lines and in cDNA panels derived from fetal and adult tissues, thus validating the microarray data. We compared anillin with Ki67 mRNA expression and found a significant linear relationship between anillin and Ki67 mRNA expression (Spearmann r = 0.6, P < 0.0001). Anillin mRNA expression was analyzed during tumor progression in breast, ovarian, kidney, colorectal, hepatic, lung, endometrial, and pancreatic tumors and in all tissues there was progressive increase in anillin mRNA expression from normal to benign to malignant to metastatic disease. Finally, we used anti-anillin sera and found nuclear anillin immunoreactivity to be widespread in normal tissues, often not correlating with proliferative compartments. These data provide insight into the existence of nonproliferation-associated activities of anillin and roles in interphase nuclei. Thus, anillin is overexpressed in diverse human tumors, but not simply as a consequence of being a proliferation marker. Anillin may have potential as a novel biomarker.

The process of cytokinesis involves a complex choreography of regulatory and structural events (1). A compelling body of data suggests that anillin is a key component that couples filament systems during cytokinesis and may influence their spatial organization (2, 3). Anillin was originally identified as an actin-binding protein by affinity chromatography (4) and its location is cell cycle regulated in cultured cells, being absent in $G_0$; accumulating in the nucleus during $G_1$, $S$, and $G_2$; and then redistributing to the cell cortex at the onset of mitosis (2, 3). With the onset of cytokinesis, anillin localizes to the cleavage furrow and forms a ring (anillos—Spanish for ring; ref. 2), where it is required for the final phases of cytokinesis (2, 3, 5).

As well as binding actin via its NH$_2$-terminal domain (2), anillin has been shown to bind at least some septins (3, 6) and to associate with myosin II in a phosphorylation-dependent manner (5).

Recent data indicate that septins have roles in a number of disease states, including in neoplasia (7, 8). Mammalian septins are involved in cytokinesis and in other processes (7) and some septins can exist in the nucleus (9). As part of our studies of septin biology, we investigated the septin-binding protein, anillin. We have used a range of methods to define the expression of anillin mRNA and protein in human tissues. We found that anillin mRNA and protein is expressed in all tissues and maximally in brain. Whereas anillin message levels correlate with Ki67 expression (a well-characterized proliferation marker whose expression is tightly linked to growth fraction; refs. 10, 11), this is not absolute and anillin immunoreactivity is present in both proliferative and nonproliferative compartments with highest levels in the brain. Furthermore, anillin mRNA levels are increased in diverse human tumors and expression increases with tumor progression.

Materials and Methods

Expression microarray experiments. The Affymetrix HG-u133 GeneChip was screened for multigolomer probes corresponding to the anillin mRNA sequence (NM_018685). The multigolomer probe set 222608_at with the highest proportion of samples called "present" was chosen for downstream analysis on 7,287 fresh frozen human tissue samples and 292 cell lines with sample preparation and data analysis done by GeneExpress (GeneLogic, Gaithersburg, MD). Relevant ethical
permissions were obtained and the samples obtained with due regard for ethical issues. Diagnoses of the human tissue samples were verified by internal review by the Department of Pathology at Genentech. A Ki67-specific probe set (212020_s_at) was used as a control data set because Ki67 expression is cell cycle regulated (10, 11). Statistical analysis was carried out using GraphPad Prism 4 software. Analysis by the Kruskal-Wallis test was done comparing the three GeneExpress database categories (normal, nonmalignant diseased, and malignant) in each tissue type. Pairwise comparisons of categories used the Dunn posttest. Spearman correlation coefficient of relevant anillin and Ki67 data sets was also calculated in each tissue type.

RNA extraction, reverse transcription, PCR, and cloning. Total RNA was extracted from cell lines by standard methods (RNA Stat-60; Tel-Test) and was subjected to RQ1 DNase digestion (Promega, Madison, WI). cDNA was generated using random hexamer priming and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Paisley, Scotland). Relevant negative controls were always included. In other experiments, adult or fetal human normal tissue cDNA panels (BD Biosciences, San Jose, CA) were used as template for PCR reactions. PCR amplification was carried out using a primer pair for anillin [F3 5'-ATGCAGGAACTCAATAACGAAA-3' and R1 5'-AGGCTTTCCAATAGGTTTGTAGCA-3'] and a primer pair for β-actin

Fig. 1. Electronic Northern blot for anillin mRNA expression in 7,287 tissue samples and 282 cell lines (green, normal; blue, diseased nontumor; and red, tumor).
[BA67 5'-GGGAGACCGGAAATCGTGCGTGACATT-3' and BA68 5'-GATGGAGTTGAAGGTAGTTTCGTC-3']. PCR products were analyzed on a 1% agarose gel and visualized with ethidium bromide. Real-time quantitative PCR was done using Absolute QPCR SYBR Green Mix (ABgene, Cambrigde, United Kingdom) on a GRI Lightcycler using a primer pair for anillin [F4 5'-TGGCTAATTGTACCAGTCGTCAGA-3' and R1 5'-AGGCTTTCCAATAGGTGTAGCA-3'] and normalized to β-actin [primer pair BA2F-P 5'-GGCTCCGGCATGTGCAAG BA3R-P 5'-CCTCGGTGCAAGCACGG]. A full-length anillin construct was generated by PCR, cloned into pECDNA3.1/V5-His-Topo, and sequenced on both strands by standard methods (Applied Biosystems, Foster City, CA).

Antibody production, characterization, and immunohistochemistry. Polyclonal anti-anillin sera (S3 and S4) were raised to keyhole limpet hemocyanin–conjugated peptides [781LKNEGPQRKNKASPQ796 and 1017YWTPDDEKRKNPIG1031] using standard methods (Eurogentec, Liege, Belgium), purified with Melon gel (Pierce Biotechnology Rockford, IL), and affinity purified by standard methods. Protein lysates derived from cell lines were resolved on polyacrylamide gels, transferred to nitrocellulose filters, and probed with relevant immune or preimmune sera. Protein bands were detected by enhanced chemiluminescence (12). Full-length anillin cDNA were expressed in coupled in vitro transcription/translation reactions was similarly resolved and detected with anti-V5 epitope tag antibody.

Cell lines (HeLa, CaOV3, OTN14, A2780, MDA-MB-361, MCF7, RKO, H630, and 293T) were grown under standard conditions. After fixation [methanol (−20°C) or 2% paraformaldehyde in PBS for 10 minutes], cells were rinsed with PBS, permeabilized in PBS with 0.1% Triton X-100, and stained with anti-anillin sera [rabbit sera S3 or S4 (above) or goat serum Ab5910 (AbCam, Cambridge, United Kingdom)]. Bound antibody was detected with FITC-conjugated anti-rabbit or anti-goat sera (DakoCytomation, Ely, United Kingdom) with a 4′,6-diamidino-2-phenylindole nuclear counterstain. Cells were visualized on a Leica FW4000 microscope system. In other experiments, pellets of cell lines (HeLa, CaOV3, A2780, MCF7, RKO, and 293T) were fixed in 10% formalin, embedded in wax, and processed for immunohistologic analysis. Immunohistochemistry was done on paraffin sections of cell pellets and normal tissue microarrays using standard methods (13) with ethical approval from the Northern Ireland Local Research Ethics Committee.

Results

Anillin mRNA is transcribed from 24 exons of the anillin gene (ANLN, NM_018685) that span 84 kb on chromosome 7p14.2. This encodes a basic (isoelectric point, 8.3) 1,125-residue protein with a predicted molecular weight of 123,477.72 Da. A single Affymetrix probe set (222608_at) maps to 3′ end of the anillin mRNA. Figure 1 shows an electronic Northern blot based on the analysis of 7,287 fresh frozen human tissue samples and 292 cell lines. Anillin mRNA is expressed in all tissues, albeit at varying levels. Given the association of anillin with cytokinesis, we compared expression of anillin with that of Ki67. There is a statistically significant, but modest, correlation between anillin and Ki67 mRNA expression (r is typically ~0.6; see Fig. 2A-E). The multiple regression coefficient (r^2) is typically ~0.36, indicating only a proportion of the variance in anillin is related to Ki67 expression and, by inference, proliferation. In the brain (Fig. 2F), this correlation is lost as there is high-level expression of anillin mRNA but Ki67 expression is negligible as would be expected because the vast majority of cells of the adult central nervous system are postmitotic.

We examined the expression of anillin mRNA in human tumor cell lines by reverse transcription-PCR (RT-PCR; Fig. 3A). PCR products of the expected size are detected in all cell lines but with varying intensity indicative of differing levels of anillin mRNA expression. This was confirmed using a real-time quantitative RT-PCR method (Fig. 3B), and the results of both assays are positively correlated (r = 0.87;
The quantitative RT-PCR method was then used to screen cDNA panels derived from fetal (Fig. 3C) and adult (Fig. 3D) normal tissues. Anillin mRNA is ubiquitously expressed in fetal development and in adult tissues, but at varying levels consistent with the data from the microarray analysis (Fig. 1). For example, these data show that anillin mRNA is highest in brain; and with high levels in testis and placenta; intermediate levels in skeletal muscle, thymus, ovary, and intestine (small and large); and lower levels in heart, brain, lung, liver, kidney, pancreas, spleen, and prostate. Comparison of the real-time RT-PCR expression levels with the median signal intensity in microarray studies show a positive linear correlation ($r = 0.81$, $P < 0.0001$), validating the microarray data.

From the complete microarray data set, samples from tissues in which tumors are not represented, or were represented in small numbers, or where there is lack of homogeneity in the categories (e.g., blood vessels, heart, head and neck, gall bladder) were excluded. This left a data set ($n = 5,856$) where anillin expression was defined by tissue, and then into normal, nontumor diseased, and tumor categories. Samples were grouped by histologic diagnosis. Ki67 mRNA expression in this series of samples is consistently elevated in tumors compared with normal tissues (see Supplementary Table S1). There is higher expression of anillin mRNA in tumors compared with normal tissue controls and the fold inductions are larger than for Ki67 (mean fold increase, 3.54 versus 1.61 for Ki67). The comprehensive nature of the GeneLogic database allowed the identification, in eight tissues, samples that reflected tumor progression with data from normal, hyperplastic, benign, malignant, and metastatic samples (Fig. 4). There is a progressive increase in median anillin mRNA expression during tumor progression in breast, colorectal, endometrial, liver, lung, renal, kidney, ovarian, and pancreatic cancer (in all cases, $P < 0.0002$ by ANOVA). Of note is that this analysis with the Ki67 data set gives a similar but less strong association with disease progression.

Anillin protein expression was analyzed in cell lines using a polyclonal anti-anillin serum and also a commercial goat anti-anillin serum (ab5910) raised to the COOH-terminal 13 residues of anillin ($^{1112}$WQPACYK PIGK$^{1125}$). Ab5910 gave no signal in Western blots. The rabbit anti-anillin serum recognizes a band of ~180 kDa in lysates of HeLa, MCF7, and RKO cells (Fig. 5A) and the signal intensity correlates with mRNA levels (Fig. 3A). The electrophoretic mobility is less than predicted as reported previously but is the same as...
that of in vitro transcription/translation–expressed anillin protein. Immunofluorescence studies of cultured cells using a polyclonal rabbit anti-anillin serum and the commercial antiserum (ab5910) show staining patterns identical to that reported in other systems (2, 3). Anillin expression was at the cytokinetic ring in telophase (Fig. 5B), but is present in the nucleus in interphase (Fig. 5C). Taken together, the data indicate that both reagents detect anillin protein in human cells.

We undertook an immunohistologic analysis of anillin protein expression in normal human tissues (Fig. 6). Both sera gave equivalent results and anillin immunoreactivity was seen in all adult tissues consistent with the mRNA data. Immunoreactivity took three forms—nuclear, cytoplasmic, or mixed.

Nuclear staining could be seen in some cells derived from all germ layers and in cells from continually renewing, conditionally renewing, and quiescent populations. Similarly, cytoplasmic immunoreactivity could be seen in an overlapping distribution. The most striking observation is the lack of clear correlation of anillin immunoreactivity with proliferative compartments. Anillin expression is marked in the central nervous system and in quiescent populations such as parathyroid, liver, salivary gland, and prostate. The amount of staining is variable with some cells showing strong nuclear expression, whereas others do not. The meiotic cells of the testis and ovary show intense nuclear anillin immunoreactivity.

With regard to cytoplasmic expression of anillin, three patterns were observed. In general, diffuse cytoplasmic staining...
was seen (e.g., fallopian tube, small intestine, liver, prostate, transitional epithelium, renal tubules, and salivary gland ducts). However, in the colon and pancreatic ducts and gall bladder epithelium, supranuclear cytoplasmic immunoreactivity was observed, which may represent staining of the Golgi apparatus. Finally, striking staining of the intercalated discs (Z disc) of the myocardium was observed, a region enriched for vinculin, neural cell adhesion molecule, and β-catenin (14). Relatively weak staining of skeletal muscle is seen and this is also membrane associated, whereas smooth muscle showed more diffuse cytoplasmic immunoreactivity.

Discussion

We present a detailed characterization by multiple modalities of the expression of anillin mRNA and protein in human cells and tissues. The data sets generated provide cross-validation and paint a clear picture of the widespread distribution of anillin mRNA and protein. Our quantitative RT-PCR analysis shows that anillin mRNA is expressed in diverse tissues and the levels correlate well with those observed by microarray analysis. There is a general, but not absolute, correlation between anillin and Ki67 mRNA expression. Our immunohistologic data indicate that anillin protein is widely expressed in diverse tissues, including in nonproliferative cells (e.g., neurons, terminally differentiated epithelial, and quiescent stromal cells), as well as in proliferative compartments. Anillin mRNA and protein expression cannot, therefore, be simply viewed as yet another marker of proliferation.

In cultured cells, anillin protein expression is predominantly, but not exclusively, nuclear, and profound spatial reorganization of anillin occurs through the cell cycle (2, 3). In G0, levels of anillin protein are negligible, consistent with the report of Whitfield et al. (11) who showed that anillin mRNA is cell cycle regulated in vitro. Indeed, anillin has been posited to be a marker of quiescent populations. The cell cycle distribution of anillin in Drosophila suggested that in fly development, G0 cells have no postmitotic cells. The cell cycle distribution of anillin in human cells and tissues. The data sets generated provide cross-validation and paint a clear picture of the widespread distribution of anillin mRNA and protein. Our quantitative RT-PCR analysis shows that anillin mRNA is expressed in diverse tissues and the levels correlate well with those observed by microarray analysis. There is a general, but not absolute, correlation between anillin and Ki67 mRNA expression. Our immunohistologic data indicate that anillin protein is widely expressed in diverse tissues, including in nonproliferative cells (e.g., neurons, terminally differentiated epithelial, and quiescent stromal cells), as well as in proliferative compartments. Anillin mRNA and protein expression cannot, therefore, be simply viewed as yet another marker of proliferation.

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It is striking that anillin is expressed widely and often in postmitotic cells. The cell cycle distribution of anillin in Drosophila suggested that in fly development, G0 cells have no detectable anillin (2). Furthermore, detailed examination of fly development suggests that anillin only localizes to the nuclei of dividing cells and may mark cells with the potential to divide. Indeed, anillin has been posited to be a marker of somatic stem cells (17, 18). Whereas this may be true of the fly, our immunolocalization studies in human tissues are not consistent with this conjecture. However, there are some parallels between flies and man because anillin is expressed at high levels in germ cells, consistent with observations of anillin being required for meiotic cytokinesis in Drosophila (17, 19).

We have found that anillin mRNA is consistently overexpressed in tumors derived from diverse sites and have shown an increase in anillin mRNA expression during tumor progression. These data are supported by other recent reports. For example, anillin is overexpressed in brain tumors and at the transition from in situ to invasive disease (20). Similarly, anillin is overexpressed in endometrial carcinomas (21), gastric cancer (22), and uveal melanoma (23). However, the mechanism of anillin overexpression in tumors is not clear. The correlation of anillin and Ki67 mRNA expression suggests that anillin expression is regulated by cell cycle–dependent factors, but this is unlikely to be the entire explanation because anillin is expressed in nondividing cells in many tissues. Another possibility is that the anillin locus might be amplified in neoplasia. Chromosome 7p harbors loci amplified in cancer such as the epidermal growth factor receptor locus at 7p12.3 but this amplicon is typically <2 Mb (24). The anillin locus at chromosome 7p14-15 is 18.5 Mb telomeric to this and is, thus, unlikely to be on the same amplicon. Furthermore, epidermal growth factor receptor overexpression is much more restricted (25) than seen with anillin. Finally, genes flanking the ANLN locus (SEPT7, 484,461 bp telomeric and AMPH, 1,929,545 bp centromeric) are not overexpressed in cancer, suggesting that either this chromosomal fragment is not amplified or, if it is, the amplicon is small. That anillin overexpression is so common perhaps suggests a more global role in cellular physiology with deregulation in neoplasia.

Other proteins associated with cytokinesis are altered in neoplasia. Kinetochore proteins, such as CENP-F, CENP-A, and Nek2 kinase, are overexpressed in tumors (25–28) with the CENP-F locus being amplified in head and neck tumors (29). The aurora kinases have been implicated in neoplasia and have altered expression in tumor progression (30) as do key elements of the cytokinesis apparatus, such as citron kinase (31, 32) and polo-like kinases (33). The key cytokinesis-associated myosin, myosin II, can be altered in neoplasia (34). As well as binding anillin (5), myosin II interacts with the metastasis-associated protein S100A4 (35) and the nuclear tumor suppressor meningi (36). Clearly, then, the molecular machinery associated with cytokinesis can be deranged in neoplasia, often with altered expression. It is also of note that patients with neoplasia may develop autoantibodies to kinetochore and other cytokinesis-associated proteins (37). It will be interesting to search for anillin autoantibodies in patients with cancer.

It has been proposed that the nuclear location of anillin in interphase is simply to keep it spatially separate from cytoplasmic components (2). It would seem more likely that anillin has active nuclear roles because pools of cytoplasmic anillin exist. Actin is present in the nucleus (38, 39) where it has diverse roles interacting with nuclear proteins including polymerases (40), helicases (41), transcriptional regulators (42), chromatin remodeling proteins (43), ribonuclear proteins (44), structural proteins (45), and tumor suppressor genes such as β53 (46). Actin may also have a role in energy-dependent, vectorial

4 P.A. Hall, unpublished data.
movement of multimolecular complexes and organelles around
the nucleus (47), and at least one myosin isoform exists in the
nucleus (48). Might anillin have roles in such actin-associated
nuclear processes? Interestingly, nuclear septins are now
recognized (9), including SEPT6, which is an anillin-binding
protein (6). Given the expression of anillin in the nucleus, anillin
functions may be important in nuclear physiology, and
overexpression of anillin may perturb the nucleus in cancer.
Anillin may also prove to be a new biomarker of cancer and
disease progression. Studies to test this hypothesis are in
progress.

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