Blastemal Expression of Type I Insulin-Like Growth Factor Receptor in Wilms’ Tumors Is Driven by Increased Copy Number and Correlates with Relapse

Rachael Natrajan, Jorge S. Reis-Filho, Suzanne E. Little, Boo Messahel, Marie-Anne Brundler, Jeffrey S. Dome, Paul E. Grundy, Gordan M. Vujanic, Kathy Pritchard-Jones, and Chris Jones

1Paediatric Oncology, Institute of Cancer Research/Royal Marsden National Health Service Trust, Sutton, United Kingdom; 2The Breakthrough Breast Cancer Research Centre, Institute of Cancer Research, London, United Kingdom; 3Birmingham Children’s Hospital, Birmingham, United Kingdom; 4Department of Paediatrics and Oncology, University of Alberta, Edmonton, Alberta, Canada; and 5University of Wales Medical School, Cardiff, United Kingdom

Abstract

Most Wilms’ tumors are of low stage, favorable histology, and have a high likelihood of cure with current multimodal therapy. Despite this, there remains a group of patients whose tumors recur for whom intensive salvage regimens result in survival of only 50%. Fitting a Cox proportional hazards model to microarray-based comparative genomic hybridization (aCGH) data on 68 Wilms’ tumor samples, we identified a significant correlation between increased copy number at chromosome 15q26.3 insulin-like growth factor I receptor (IGFIR) and tumor relapse (adjusted P = 0.014). Wilms’ tumors (13%) exhibited a low-level gain corresponding to three to four copies of the gene by aCGH analysis, 9 of 10 of which exhibited high IGFIR mRNA levels. Although IGFIR protein expression was restricted to the epithelial cells of fetal kidney and Wilms’ tumors in most cases, 12% of tumors were also found to express IGFIR in the blastemal compartment. Blastemal IGFIR protein expression was associated with an increased copy number and a shorter relapse-free survival time (P = 0.027, log-rank test). In addition to the membrane localization, IGFIR was localized to the perinuclear region of the blastemal cells in 6% of Wilms’ tumors. These data provide evidence that an increase in IGFIR gene copy number results in aberrant expression in the blastemal compartment of some Wilms’ tumors and is associated with an adverse outcome in these patients. These findings suggest the possibility of use of targeted agents in the therapy of these children. (Cancer Res 2006; 66(23): 11148-55)

Introduction

Wilms’ tumor (nephroblastoma) is the most common pediatric renal malignancy and is viewed as a prototype of differentiation failure in human neoplasia as it recapitulates the histology of the nephrogenic zone of the growing fetal kidney. With improved multimodality therapy, survival has risen over the last 40 years to 85% to 90%; however, for those whose disease relapses, even intensive salvage regimens result in subsequent survival closer to 50% (1). Genes involved in nephrogenesis, and particularly the mesenchymal to epithelial transition that accompanies the ingrowth of the ureteric bud into the metanephric blastema, have also been implicated in Wilms’ tumorigenesis; however, to date, there are no specific genes known to be involved in tumor aggressiveness.

Treatment of Wilms’ tumors is currently based on anatomic tumor extent (stage) and histologic subtype. The most important adverse prognostic indicator with respect to histology in untreated Wilms’ tumor is the presence of anaplasia (“unfavorable” histology; ref. 2). Defined by the presence of marked nuclear enlargement, hyperchromasia, and multipolar polyplody mitotic figures, anaplastic Wilms’ tumors are strongly associated with somatic TP53 mutations (3). Anaplasia can be focal or diffuse, the latter subtype having survival rates of only ∼50%. However, because anaplastic tumors account for only 5% to 10% of all Wilms’ cases, the majority of relapses are in the favorable histology group, suggesting involvement of other genetic factors. In tumors pretreated with chemotherapy before resection, the persistence of the blastemal component has also been implicated as a marker of poor outcome (4).

Somatic mutations of WT1, at 11p13, occur in 10% to 15% of sporadic tumors (5) and are associated with a stromal-predominant histology. However, they do not seem to correlate with clinical outcome. Somatic WT1 mutation abnormalities correlate strongly with β-catenin mutation, with approximately half of the WT1 mutant tumors also harboring activating β-catenin mutations (6). In contrast to its normal cytoplasmic localization, nuclear localization of β-catenin has been reported in Wilms’ tumors even in the absence of an activating mutation (7). A second Wilms’ tumor locus at 11p15 contains several imprinted genes and exhibits a variety of alterations constitutionally in patients with Beckwith-Wiedemann syndrome (associated with somatic overgrowth and embryonal tumors, including Wilms’; ref. 8). One gene whose expression is commonly increased by these alterations is insulin-like growth factor (IGF) II (IGFII), which encodes an embryonal growth factor (9, 10).

IGFs signal through the IGF1 receptor (IGFIR), a tyrosine kinase cell surface receptor that binds either IGF-I or IGFII (11). Among multiple IGFIR substrates, insulin receptor substrate-1 (IRS-1) and phosphatidylinositol 3-kinase (PI3K) have been reported to play a role in IGFIR-mediated cell proliferation and cell protection from apoptosis via activation of the Ras/mitogen-activated protein kinase (MAPK) pathway and phosphorylation of Akt/protein kinase B (12). Functional IGFIR may also have a key role in the survival of transformed cells by inhibiting apoptosis (13). IGFIR further acts in...
the regulation of cell-cell and cell-matrix adhesion, colocalizing and forming a complex with E-cadherin/β-catenin (14). Intriguingly, IGFIR induces rapid β-catenin relocation to the nucleus in epithelial to mesenchymal transition (15), and one mechanism of regulation of nuclear translocation of β-catenin seems to be via IGFIR-mediated interaction with IRS-1 (16).

Previous work in our laboratory has used microarray-based comparative genomic hybridization (aCGH) to identify copy number changes associated with relapse in favorable histology Wilms’ tumor (17). Here, we present the identification of copy number changes associated with relapse in favorable histology Wilms’ tumors taken at immediate nephrectomy with full clinical follow-up data, enriched for those which recurred, and has been reported previously (17). Samples were taken at surgery and frozen in liquid nitrogen. Genomic DNA was extracted using a standard proteinase K digestion followed by phenol/chloroform extraction and resuspended in water.

**Materials and Methods**

**Sample collection.** Primary Wilms’ tumor samples were obtained after approval by local and multicenter Ethical Review Committees from the North American Children's Oncology Group and the United Kingdom Childhood Cancer Study Group (UKCCSG). The sample set consisted of 68 favorable histology Wilms’ tumors taken at immediate nephrectomy with full clinical follow-up data, enriched for those which recurred, and has been reported previously (17). Samples were taken at surgery and frozen in liquid nitrogen. Genomic DNA was extracted using a standard proteinase K digestion followed by phenol/chloroform extraction and resuspended in water.

**Microarray CGH.** The 68 Wilms’ tumors were analyzed on the Breakthrough Breast Cancer human CGH 4.6K 1.1.2 arrays (Array Express accession number A-MEXP-192) consisting of 4179 BAC clones, spaced at ~1 Mb throughout the genome and spotted in triplicate onto Corning GAPPSII-coated glass slides (Corning, NY; ref. 18) as reported previously (17). The final data set comprised 68 samples and 3,999 clones. All data have been submitted according to MIAME guidelines (19) to the public data repository ArrayExpress with accession number E-TABM-10.

**Statistical analysis.** All data transformation and statistical analysis were carried out in R 2.0.18 and BioConductor L5.3, making extensive use of modified versions of the package aCGH in particular (20). For each individual BAC clone on the array, a Cox proportional hazards model was fitted with respect to relapse-free survival. This was carried out both in a univariate manner, as well as multivariate, incorporating tumor stage into the model. To correct for multiple testing, the step-down permutation procedure maxT (21) was done, providing strong control of the family-wise type I error rate.

**Fluorescence in situ hybridization mapping and end sequencing of BAC clones.** Amplification of BAC DNA was done using the GenomiPhi Whole-Genome Amplification kit (GE Healthcare, Amersham, United Kingdom) according to the manufacturer’s instructions. Probe fluorescence in situ hybridization (FISH) mapping and end sequencing were carried out as described previously (22). Labeled probes were hybridized to metaphase spreads (Vysis Inc., Downers Grove, IL) and analyzed with CytoVision software (Applied Imaging International, Newcastle upon Tyne, United Kingdom). The genomic locus of each BAC clone was determined according to the UCSC BAC End Pair algorithm.10

**Quantitative real-time reverse-transcription-PCR.** cDNA was prepared from 1 μg tumor or reference RNA by random primed reverse transcription using SuperScript II (Invitrogen, Paisley, United Kingdom). IGFIR Assay on Demand (Hs.00181385) was obtained from Applied Biosystems (Warrington, United Kingdom). PCRs were done in a 10-μl reaction volume containing 5 μl 2× buffer/enzyme mix, 0.5 μl 20× assay mix, 0.5 μl 20× glyceraldehyde-3-phosphate dehydrogenase endogenous control assay mix (Hs.9999905), and 1 μl input cDNA. Assays were run on an Applied Biosystems 7900 Sequence Detection System and results were analyzed by the standard curve method. Data were normalized to Universal Human Reference RNA (Stratagene, La Jolla, California).

**Immunohistochemistry.** Immunohistochemistry was done on 5-μm formalin-fixed, paraffin-embedded sections using a mouse monoclonal antibody to human IGFIR (AB4065, Abcam, Cambridge, United Kingdom), using the Envision-horseradish peroxidase system (K4006, DAKO, Ely, United Kingdom) at a dilution of 1:25 according to the manufacturer’s instructions. Antigen retrieval was done by boiling the slides in 1 mmol/L EDTA (pH8.0) in a microwave for 15 minutes.

**Tissue microarrays.** Pediatric renal tumor tissue microarrays were constructed (23) containing replicate representative cores (n = 885) from all available cellular components from 274 Wilms’ tumors, 14 clear cell sarcomas of the kidney (CCSK), 9 mesoblastic nephromas (MN), and 7 rhabdoid tumors of the kidney (RTK). Tumors were treated either according to National Wilms’ Tumor Study Group (NWTSG) guidelines (immediate nephrectomy) or Society of Paediatric Oncology protocols (delayed nephrectomy following preoperative chemotherapy). There was a slight enrichment of tumors that relapsed. The presence of tumor tissue on the arrayed samples was verified on a H&E-stained section. Tumor cell positivity, cellular distribution, and subcellular localization were assessed independently by three pathologists (J.S.R-F., M-A.B., and G.M.V.).

**Results**

Copy number gain at chromosome 15q26.3 correlates with shorter relapse-free survival time in favorable histology Wilms’ tumors. To identify novel loci that correlated with poor clinical outcome in favorable histology Wilms’ tumors, we applied survival statistics to our genome-wide copy number data. For each clone on the array, we independently fitted a Cox proportional hazards model with respect to relapse-free survival. To control for false positives, permutation-based correction for multiple testing was carried out. Figure 1A shows those clones which were associated with relapse with an adjusted P < 0.05, plotted in genome order. Such an approach identified loci at 1q, 12q, and 21q as identified previously by class comparison; however the most significant associations, with the highest Cox scores (6.173) and lowest adjusted P values (P = 0.014) were with loci on chromosome 15q26.3. To further investigate the clinical associations, tumors were categorized as either containing gain at this locus or showing normal copy number, and the association with relapse-free survival was confirmed by doing a Kaplan-Meier analysis (P = 0.022, log-rank test; Fig. 1B). In our sample set, 9 of 68 (13%) tumors exhibited gain at 15q26.3 (Fig. 1C). This was observed as a focal gain of two or three clones, with log2 ratios approximating to three to four copies, as determined on our platform by FISH analysis (17). To ensure the correct genomic location of these clones, end sequencing and FISH mapping were carried out (Fig. 1D). The clones RP11-308P12 and RP11-397C10 were confirmed as mapping...
Figure 1. Copy number gain at chromosome 15q26.3 correlates with shorter relapse-free survival time in favorable histology Wilms’ tumors. A, Cox proportional hazards model with respect to relapse-free survival time applied to the aCGH data. Y axis, Cox score; X axis, clones according to genomic location. Only those clones with an adjusted \( P < 0.05 \) are plotted. The top-ranked clones are found chromosomes 1q, 12q, and 15q (arrow). B, Kaplan-Meier plot for tumors with and without gain at 15q26.3 with respect to relapse-free survival. C, representative aCGH plots for two tumors with focal copy number gain at 15q26.3 (highlighted). Y axis, \( \log_2 \) ratios; X axis, clone according to genomic location. Vertical dotted line, centromere; horizontal dashed lines, \( \log_2 \) ratios of 0.15 and \(-0.15\). D, FISH mapping of clone RP11-308P12 confirmed location on 15q26.3. End sequencing of clones RP11-308P12 and RP11-397C10 showed mapping to the \( \text{IGFIR} \) locus.
to a 1.25-Mb region between 96, 148, 980 bp and 97, 405, 140 bp, encompassing two expressed sequence tags (ARRDC4 and FLJ3974c) and a single known gene, the IGFR (IGFIR).

**IGFIR is gained and overexpressed in Wilms’ tumors and correlates with relapse.** To specifically investigate expression of the IGFR gene in Wilms’ tumors, quantitative real-time reverse transcription-PCR (RT-PCR) was carried out on a series of 71 samples, 38 from our original aCGH set and 33 from an independent cohort. IGFR expression was found to be rather variable across our Wilms’ tumor samples, with expression levels varying >40-fold between the low and high expressers (Fig. 2A). High levels of expression (defined as >2-fold increase of basal levels) was observed in 33 of 49 (67.3%) favorable histology cases, as well as 18 of 22 (81.8%) anaplastic Wilms’ tumors. The difference in IGFR mRNA levels was only of borderline significance when comparing relapsing tumors compared with nonrelapsing (P = 0.052, t test; Fig. 2B). To investigate the associations between DNA copy number and expression, samples with both RT-PCR and aCGH data were coplotted. Although high levels of IGFR expression were seen in 13 of 24 (54.2%) tumors in the absence of 15q26.3 copy number gain, 9 of 10 (90%) of the tumors with copy number gain also exhibited high levels of mRNA expression (P = 0.024, Fisher’s exact test; Fig. 2C). Favorable histology tumors exhibiting both increased copy number and high expression levels were found to have a shorter relapse-free survival time than those with normal expression levels/copy number or elevated gene expression alone (P = 0.0431, log-rank test; Fig. 2D).

**IGFIR is expressed in the epithelial cells of normal kidney and Wilms’ tumors but not in the metanephric blastemal cells during nephrogenesis.** The IGFR gene encodes a membrane-bound receptor tyrosine kinase. We applied immuno-histochemistry with a monoclonal antibody directed against the protein to determine its expression patterns in normal kidney and Wilms’ tumor. In the fetal kidney, the metanephric blastemal cells, from which Wilms’ tumors are thought to arise, were universally negative for IGFR protein expression (Fig. 3A). Weak to strong membranous staining was seen in the epithelial cells of the developing tubules within these fetal kidney samples (Fig. 3B). In the mature pediatric kidney, very strong receptor expression was observed in the epithelial cells of the proximal tubules, with only very weak staining in the distal tubules (Fig. 3C). Other kidney cells were negative. In Wilms’ tumors, which are typically composed of varying amounts of epithelial, blastemal, and...
stromal cells, the epithelial component was almost universally positive (Fig. 3D).

**IGFIR expression in the blastemal cells of Wilms' tumors is driven by increased copy number and correlates with poor clinical outcome.** Although receptor expression was largely confined to the epithelial cells, some positivity was noted in the blastemal and stromal cells. We investigated this further by assessing the protein expression in a pediatric renal tumor tissue microarray consisting of 274 Wilms' tumors, 14 CCSK, 9 MN, and 7 RTK. In assessable cores, positive membranous staining was observed in the blastemal cells of 37 of 135 (27.4%) favorable histology Wilms' tumors (Fig. 4A and B) and in the stromal cells in 16 of 104 (15.4%). This is probably an overestimate due to the exclusion from this calculation of tumors with no assessable blastemal cells represented in the tissue array. As a percentage of all the Wilms' tumors assessable on the arrays, we observed 12.4% blastemal positivity. In addition, four of eight (50%) anaplastic Wilms' tumors showed IGFIR expression the blastemal component. Furthermore, 0 of 13 CCSKs, 1 of 7 MN, and 2 of 6 RTKs were also IGFIR positive. To determine the role played by DNA copy number in these samples, we carried out CISH analysis on sequential sections with IGFIR-specific and chromosome 15 centromeric probes. In assessable cases, 14 of 16 (87.5%) Wilms' tumors with blastemal protein expression also exhibited an increase in copy number of the IGFIR gene, with a modal copy number of 4 (range, 3-5; Fig. 4C and D).

The samples represented on the tissue microarrays consisted of two distinct cohorts—those patients who had been treated with preoperative chemotherapy and those by immediate nephrectomy. These differing treatment regimens were analyzed separately for clinicopathologic correlations, although the proportion of blastemal IGFIR-positive tumors was similar in both (25.2% immediate nephrectomy versus 31.4% preoperative chemotherapy, no significant difference, Fisher's exact test). For tumors treated with immediate nephrectomy, blastemal expression of IGFIR was significantly associated with a shorter relapse-free survival time by univariate analysis ($P = 0.0487$, log-rank test; Fig. 4E). When a multivariate model was fitted incorporating tumor stage, IGFIR positivity was not found to be associated with outcome ($P = 0.76$, Cox proportional hazards model). Tumor samples with assessable blastemal cells after preoperative chemotherapy had a significantly reduced relapse-free survival time compared with those from untreated Wilms' tumors ($P = 0.011$, log-rank test), presumably reflecting a selection for the newly recognized poor prognosis "blastemal type" of chemotherapy-resistant cells. Although there was a clear trend toward a shorter relapse-free survival time with blastemal IGFIR positivity, this was not statistically significant in this sample set enriched for poor outcome tumors ($P = 0.114$, log-rank test; Fig. 4F). There was no direct correlation with the "blastemal type." There were no correlations between IGFIR blastemal positivity and overall survival, tumor stage or age at diagnosis, regardless of treatment protocol.

**IGFIR localized to the perinuclear region is an independent prognostic factor in Wilms' tumors.** As well as the cell membrane expression of the receptor noted in the blastemal cells of a proportion of Wilms' tumors (above), we also observed IGFIR expression localized to the perinuclear region in 8 of 135 (5.9%) Wilms' tumors (Fig. 5A and B). All of the tumors were of favorable histology. Tumors with this perinuclear localization were found to have a significantly shorter relapse-free survival time compared with those Wilms' tumors without perinuclear localization by univariate analysis ($P = 0.044$, log-rank test; Fig. 5C). Although the numbers of positive cases became small when split by treatment protocol, this correlation was evident as a trend for the immediate nephrectomy cases ($P = 0.088$, log-rank test) and as statistically significant for patients treated with preoperative chemotherapy ($P < 0.001$, log-rank test). When a multivariate model was fitted to include tumor stage and exposure to preoperative chemotherapy, perinuclear localization of IGFIR was found to be an independent prognostic marker ($P = 0.039$, Cox proportional hazards model; Fig. 5D).
Discussion

Signaling through the IGFIR, following the binding of ligands IGFI or IGFII, is widely reported to be of fundamental importance to tumorigenesis through diverse effects, including regulation of cell proliferation, survival, differentiation, and transformation (11, 12, 26). IGFII is overexpressed in more than half of Wilms’ tumors, usually as a result of loss of imprinting (9, 10). Although IGFII is normally only transcribed from the paternal allele, this maternal imprinting is lost, leading to biallelic expression of the gene. Increased signaling through the IGFIR is an obvious possible consequence of this epigenetic mechanism in Wilms’ tumor cells, and the presence of active IGFIR has been shown previously in Wilms’ tumor extracts (27).

In this study, we have identified a low-level gain (three to four copies) of the IGFIR gene, present in 13% of the Wilms’ tumors in our sample set, to be associated with tumor relapse. No high level amplifications were observed. Somatic gain or amplification of IGFIR is uncommon in human cancer although has been reported in 2% to 3% of primary breast cancers (28, 29) and 4% to 6% of rhabdomyosarcomas (30, 31). Such an amplification has not been reported previously in Wilms’ tumors, although two case reports have described constitutional low-level increase in copy number at the IGFIR locus accompanied by postnatal overgrowth and predisposition to Wilms’ tumors. One patient exhibited tetrasomy for the distal 15q24.3-qter region and Wilms’ tumor development at 6 years old (32) and another case with de novo supernumerary marker chromosome derived from an inverted duplication of chromosome 15q26.3 presented at 4 years with bilateral Wilms’ tumors (33).

High levels of IGFIR mRNA were also observed in association with increased copy number, and the concurrent gain/over-expression also correlated with tumor relapse. An earlier report showed a 5.8-fold elevation in IGFIR mRNA in Wilms’ tumors compared with normal mature kidney, and the highest levels seen in WT1 mutant tumors (34). Wild-type WT1 was later found to repress IGFIR promoter activity (35). It is of note that in our study, IGFIR copy number gain was not associated with WT1 mutation (data not shown). Expression of the receptor protein was noted in the epithelial cells of the proximal tubules in the mature kidney as well as the epithelial component of Wilms’ tumors themselves. Of more pathogenic relevance is the absence of IGFIR expression in the metanephric blastemal cells of the fetal kidney, from which Wilms’ tumors are thought to arise, and the aberrant over-expression in the blastemal compartment of at least 12% of Wilms’ tumor cohort, driven by an increase in copy number as determined
by specific CISH probes. This blastemal expression was noted to correlate with treatment failure.

We have therefore showed an association with Wilms’ tumor relapse for IGFIR at the genome, transcript, and protein levels. Moreover, we have provided evidence for the direct influence of one upon the other. It is clear from our detailed molecular pathologic analysis that high level of expression in the epithelial cells of normal kidney and Wilms’ tumors, as observed with most adult carcinomas, is not the whole story. Rather, a low-level increase in DNA copy number seems to be sufficient to up-regulate expression of the receptor protein in the blastemal compartment, a cell type in which IGFIR expression is absent in the tissue of origin of Wilms’ tumors, the metanephric kidney. This up-regulation then seems to play some role in treatment failure.

An attractive mechanism by which an up-regulation of IGFIR protein in Wilms’ tumors corresponds with treatment failure and poor outcome is the formation of an autocrine loop with tumor-derived overexpression of IGFII. It is of note that the blastemal IGFIR protein expression was noted to be predominantly focal in nature, with only relatively small islands of positivity even in dense sheets of blastema. This is intriguing given a recent study showing a mosaic allelic epigenetic heterogeneity of IGFII expression in Wilms’ tumors by allele-specific in situ hybridization (36). It is unknown whether there is a correlation between cells overexpressing IGFII and IGFIR; however, it is tempting to speculate that in cases where such a mosaicism overlaps, such subclones may prove to be particularly aggressive and/or treatment resistant and may be selected for by chemotherapeutic strategies.

We also observed an unusual perinuclear localization of IGFIR protein in 6% of cases. This aberrant localization has not to our knowledge been reported previously either for IGFIR (in any tumor type) or in Wilms’ tumor (for any protein). Perinuclear IGFIR expression was found to correlate with shorter relapse-free survival independent of tumor stage and treatment protocol. The biological significance of this localization is unknown but raises the possibility of abrogated post-translational packaging through the Golgi apparatus or even the presence of IGFIR mutations. Attempts at sequencing these cases were unsuccessful as the DNA was available only from formalin-fixed paraffin-embedded blocks, so this observation remains unexplained.

Strategies that down-regulate IGFIR, by either gene disruption, neutralizing antibodies, dominant-negative mutants, or small-molecule inhibitors, have been found to interfere with cell growth and proliferation in a variety of human cancer cell types. Indeed, an early study showed the ability of an antibody directed against IGFIR to inhibit growth of Wilms’ tumor cells in culture and in athymic mice (37). Modern small-molecule inhibitors NVP-AEW541 and NVP-ADW742 show in vitro activity against diverse tumor cell types, even those resistant to conventional therapies (38, 39). These agents have also been shown to sensitize tumor cells to other anticancer agents (including doxorubicin, frequently used in later-stage Wilms’ tumors) in vitro and in vivo. Abrogation of IGFIR signaling after cytotoxic therapy may enhance the efficacy of conventional therapies by removing key antiapoptotic signals.

In summary, we have identified a low-level increase in copy number of the IGFIR gene to be significantly associated with treatment failure and relapse in Wilms’ tumor. Coupled with autocrine activation of IGFIR by IGFII produced in large amounts in some tumors as a result of loss of imprinting, this may represent a novel mechanism by which an increased mitogenic/antiapoptotic action may be derived through PI3K/Akt/S6K and/or Ras/MAPK signaling pathways as well as potentially activating β-catenin-mediated transcription through its nuclear translocation. This observation opens up the exciting possibility for novel
strategies targeting the IGFIR, which may be beneficial in relapsed Wilms’ tumor patients, either alone or in combination therapies.

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References

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