

RESEARCH ARTICLE

Expression of NRP-1 and NRP-2 in Endometrial Cancer

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Abstract: Background: Neuropilins (NRPs) participate in many processes related to cancer development such as angiogenesis, lymphangiogenesis and metastasis. Although endometrial cancer is one of the most common gynecological cancers, it has not been studied in terms of NRPs expression.

Objective: The aim of this study was to investigate the potential utility of NRPs as important factors in the diagnosis and treatment of endometrial cancer.

Method: Our study consisted of 45 women diagnosed with endometrial cancer at the following degrees of histological differentiation: G1, 17; G2, 15; G3, 13 cases. The control group included 15 women without neoplastic changes. The immunohistochemical reactions were evaluated using light microscopy.

Results: We did not detect the expression of NRP-1 and NRP-2 in the control group. NRP-1 expression was found exclusively in cancer cells. It was higher in G2 and G3 and reached about 190% of G1. NRP-2 expression was observed in the endothelium and was similar across all three cancer grades. In cancer cells, NRP-2 expression increased with the degree of histological differentiation.

Conclusion: NRP1 and NRP2 are candidates for complementary diagnostic molecular markers and promising new targets for molecular, personalized anticancer therapies.

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1. INTRODUCTION

Neuropilins (NRPs) have been identified as class 3 semaphorin co-receptors responsible for axon guidance and the development of the nervous system [1-4]. In neurons and other cell types, they form a complex with specific plexins [5,6]. Studies have shown that NRPs are also co-receptors of vascular endothelial growth factor (VEGF) [7, 8]. NRP-1 interacts with VEGF-A and its receptor VEGFR-2. NRP-2 is a co-receptor for VEGF-C and D and interacts with VEGFR-3 [9, 10].

NRPs play a major role in signal transduction due to their ability to interact with multiple tyrosine-kinase-associated

receptors and integrins. It has been shown that NRPs interact with transforming growth factor beta (TGF- β) [11], hepatocyte growth factor (HGF) [12], and platelet-derived growth factor (PDGF) [13, 14]. NRP expression has been demonstrated in many tumors such as ovarian, pancreatic, and prostate cancer, as well as in astrocytoma and many other malignancies [4, 10, 15-17], which can be primarily associated with increased angiogenesis and tumor cell survival [8]. It has been shown that elevated expression of NRP-1 correlates with increased invasiveness and shortened patient survival, whereas NRP-2 expression correlates with decreased apoptosis and faster tumor growth [15]. Expression of NRPs in tumors is not only associated with the presence of these proteins on the surface of endothelial cells but also on the surface and in the cytoplasm of tumor cells [18, 19]. VEGF and therefore NRPs affect the function of immune cells present in the tumor microenvironment, which in turn affects the

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host's response to cancer [20]. VEGF receptors also regulate tumor fibroblasts activity in the tumor stroma [21].

New techniques used in cancer treatment are based on inactivation or blocking of tumor-specific proteins of endothelial tumors or tumor cells [22]. With the help of these approaches, the side effects of cancer treatment can be significantly reduced, therefore attention should be paid to the identification of tumor-specific proteins.

Although endometrial cancer is one of the most commonly diagnosed cancers of the female reproductive system, occurring predominantly in postmenopausal women [23, 24], it has not been studied in terms of NRPs expression. In our previous work, based on gene expression profiling, we have suggested NRP-2 to be an important factor in the diagnosis and treatment of endometrial cancer [25]. In this study, we aim to confirm this hypothesis at the protein level.

2. MATERIALS AND METHODS

The study material consisted of endometrial tissue samples taken from patients who underwent a hysterectomy. 45 women diagnosed with endometrioid endometrial cancer constituted a study group. Pathomorphological inclusion criteria included endometrium in the proliferative phase and endometrioid endometrial adenocarcinoma at different degrees of cellular differentiation (G1-G3). Exclusion criteria from the study group were as follows: non-endometrioid endometrial cancer, endometriosis or adenomyosis, adenocarcinoma with squamous elements, coexisting cervical carcinoma, the use of hormone therapy 24 months before surgery and extreme obesity (BMI > 40). After the histopathological assessment, the study group was divided according to the degree of histological differentiation: G1, 17; G2, 15; and G3, 13. The control group included 15 women with proliferative endometrium, who were not diagnosed with neoplastic changes during routine gynecological examinations. The study included women who underwent a hysterectomy due to diagnosed endometrial cancer. Before immunohistochemical analysis, HE staining was performed.

This study was approved by the Bioethical Committee of the Medical University of Silesia, no. KNW/0022/KB/237/16.

2.1. Immunohistochemistry

The paraffin blocks provided by the Laboratory of Pathomorphology of Beskid Center of Oncology in Bielsko-Biala were used to prepare the tissue sections for immunohistochemical staining with mouse anti-NRP-1 monoclonal antibody and rabbit anti-NRP-2 polyclonal antibody (Novus Biological). Sections (5 μ m) were mounted on silane-coated slides, dewaxed in xylene and rehydrated in graded alcohols. To retrieve the antigens, the slides were incubated in citrate buffer (10 mM, pH 6.0) at 95°C for 30 min in water bath and then cooled for 30 min. Non-specific antibody binding sites were blocked with 1% BSA in PBS for 30 min at room temperature. After removing the solution containing BSA, a primary antibody (NRP-1, 30 μ g/ml or NRP-2, 0.5 μ g/ml) was applied on the slides and was incubated for 20h at 4°C. After washing in PBS-Tween 20, biotinylated secondary antibodies were applied followed by avidin-biotinylated per-

oxidase complex (Vectastain Elite ABC Kit, Vector Laboratories). To visualize the bound antibodies, diaminobenzidine (DAB) was used according to the manufacturer's recommendation. The slides were then stained with Gill's hematoxylin, dehydrated and sealed. Sections in which the primary antibody was replaced by rabbit or mouse IgG at the same concentration constituted the negative controls.

The immunohistochemical reaction was assessed with Nikon Eclipse E200 light microscope with Nikon DS-Fi1 digital camera. Three slides were prepared from each paraffin block and five pictures under 200x magnification were taken for each of them. The intensity of staining was measured as the optical density of the reaction product using the image analysis program NISAR (Nikon). The average optical density was also calculated.

2.2. Statistical Analysis

Kolmogorov–Smirnov test was used to confirm the normality of data distribution. The one-way ANOVA was performed to evaluate the statistical significance of the differences between mean variables ($p < 0.05$). The values are presented as means \pm standard deviation. Statistical analysis was conducted using the Statistica 12 PL software (StatSoft, Tulsa, Oklahoma, USA).

3. RESULTS

3.1. HE Staining

The Fig. (1) shows the results of HE staining before performing the immunohistochemical staining to better explain the histopathological changes of expression NRP-1 and NRP-2 in endometrial cancer depending on its grade and in control cells (Fig. 1).

3.2. NRP-1

The expression of NRP-1 was not detected in the control group consisting of healthy endometrium. The reaction was localized only in the cytoplasm of the cancer cells (Fig. 2). The level of this expression was low in G1 grade (Table 1). NRP-1 expression was higher in G2 and G3 than in G1 grade and reached about 190% of G1 (Table 1).

3.3. NRP-2

Similar to NRP-1, NRP-2 expression was not observed in the control (Fig. 3). In the material collected from tumors, it was found that the reaction was localized in the vascular endothelium and tumor cells.

3.3.1. Vascular Endothelium

The expression of NRP-2 in G1 and G2 was at the same level. In G3, the expression was higher but not statistically significant (Table 1).

3.3.2. Cancer Cells

NRP-2 expression in G1 cancer cells was low and constituted approximately 50% of the expression observed in endothelial cells (Table 1). The reaction was localized only in the cytoplasm (Fig. 2). In G2 cells, it was found that NRP-2

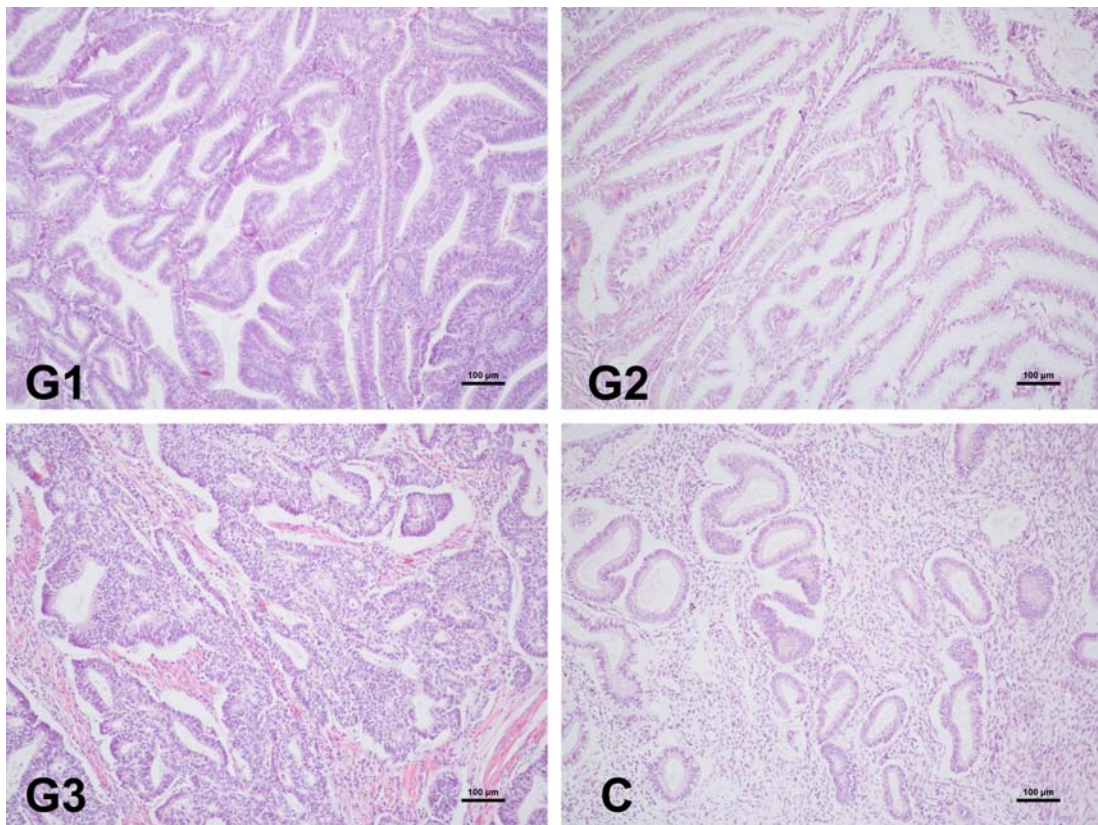


Fig. (1). The results of HE staining before the immunochemical staining in endometrial cancer depending on its grade and in control cells. G, grade; C, control. 200x magnification.

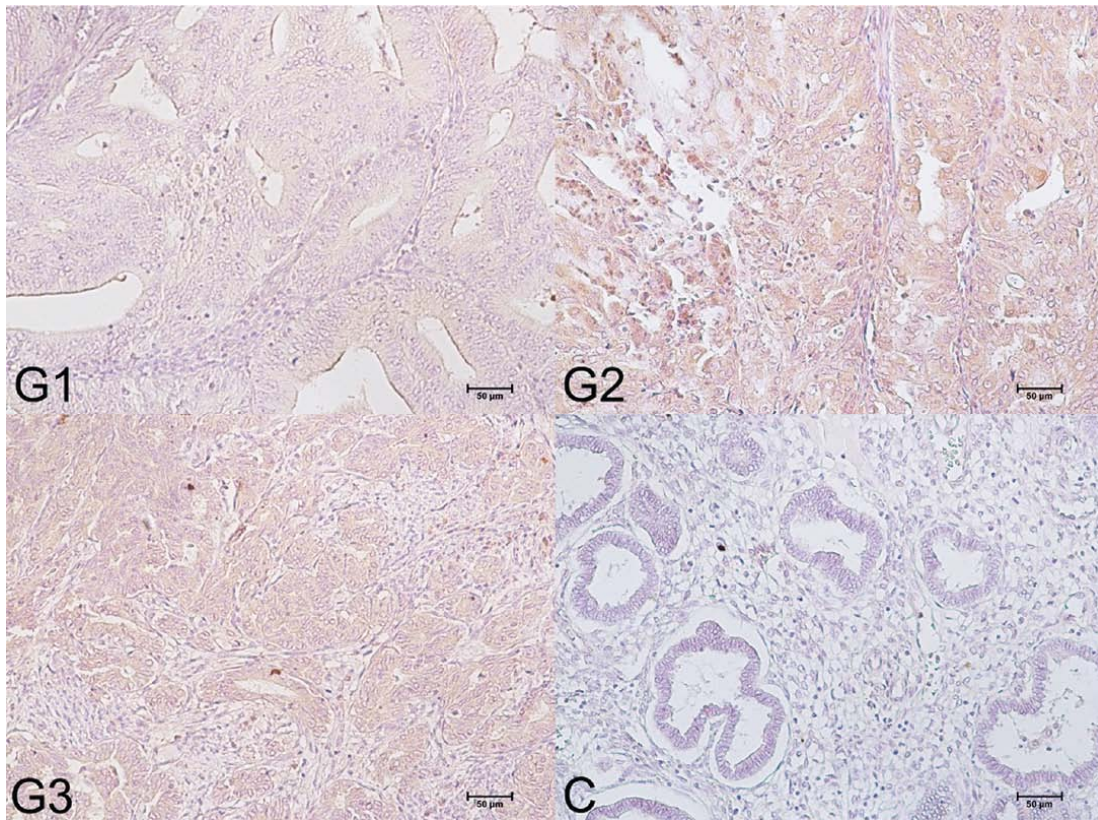


Fig. (2). Immunohistochemical localization of neuropilin 1 in endometrial cancer depending on its grade and in control cells. G, grade; C, control. Brown color indicates a positive reaction. 200x magnification.

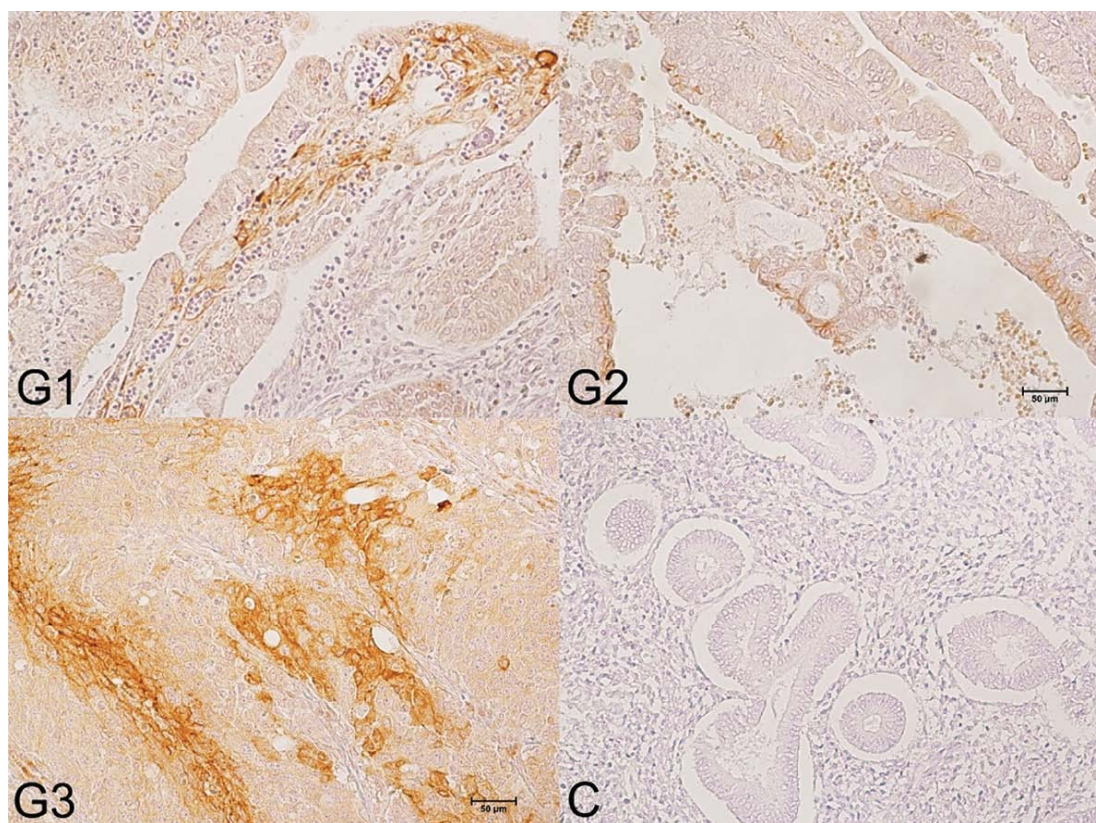


Fig. (3). Immunohistochemical localization of neuropilin 2 in endometrial cancer depending on its grade and in control cells. G, grade; C, control. Brown color indicates a positive reaction. 200x magnification.

Table 1. Optical density of the reaction product for neuropilins (NRPs) depending on endometrial cancer grade.

Protein	Localization	G1	G2	G3
NRP-1	Tumor cells	91.22 ± 6.57	173.51 ± 14.10 ^a	162.5 ± 15.73 ^b
NRP-2	Endothelium	178.32 ± 11.47	171.67 ± 16.67	197.32 ± 17.74
	Tumor cells	95.72 ± 7.72	110.83 ± 9.23 ^a	135.35 ± 12.78 ^{b,c}

Letters indicates statistically significant changes at $p < 0.05$ between:

- a = G1 and G2.
- b = G1 and G3.
- c = G2 and G3.

expression was higher than in G1 and reached about 120% of the value noted in G1 (Table 1). Some cell populations showed a cytoplasmic reaction and strong membrane reaction.

The expression of NRP-2 in G3 was found to be the highest among the studied groups (Table 1). The change was statistically significant both in relation to G1 and G2. The cytoplasmic reaction was detected in cancer cells. The cells with very strong membrane reactions were found and their amount was much higher than that in G2 cancer cells (Fig. 3).

4. DISCUSSION

Endometrial cancer is one of the most commonly diagnosed gynecological cancers worldwide. Incidence rate and high mortality force a continuous search for new therapies

characterized by greater efficacy and reduced side effects, which prolong survival and improve patient's comfort. At the same time, there is a great interest in constant search for markers that allow early detection of malignancies.

Detection of NRPs expression on the surface of endothelial cells, but especially on cancer cells, indicates that they play a particularly important role in tumor biology. These multifunctional, transmembrane glycoproteins, enable rapid growth of tumors. Binding to various proteins such as VEGF, TGF- β , semaphorins [4], as well as integrins [26] not only helps in the development of blood and lymphatic vessels, but also helps in the anti-apoptotic effects, influences the activity of T_{reg} lymphocytes and prevents host defense. Participation in epithelial-mesenchymal transition (EMT) provides migration and metastasis of the tumor [10].

Table 2. The fold change in transcriptional activity of *NRP-1* and *NRP-2* in endometrial cancer depending on its grade compared to control.

mRNA	G1	G2	G3
<i>NRP-1</i>	+1.3699117	+1.1085562	+1.0383615
<i>NRP-2</i>	+1.8524802	+1.2890337	+1.2254428

Expression or overexpression of NRPs, primarily NRP-2, has been associated with increased vascularity and poor survival [27, 28], as well as node metastases [29]. Many studies have been focused on NRPs expression in cancer, however, little is available regarding the expression of NRP in endometrial cancer.

In our previous study, we have shown that among genes that may affect the angiogenesis in endometrial cancer, significant changes in mRNA expression can be attributed to *NRP-2* (G1 vs. C, FC = +1.8542), indicating its potentially important role as a pharmacological target in the treatment of this cancer [25]. It is determined that the direction of changes of NRP-1 and NRP-2 on both mRNA and protein levels was the same. In previous work and in this study overexpression of NRP-1 and NRP-2 can be observed in all of 3 grades of endometrial cancer (Table 2).

The combination of molecular analysis methods at the transcriptome [25] and proteome levels allows us to fully observe the changes in the expression of the analyzed genes and their protein products. The same direction of changes in the expression profile of NRPs analyzed by microarray technique and immunohistochemical staining seems to indicate that protein expression can be predicted based on the transcriptional activity of the gene. Regarding NRP-1 and NRP-2, our observations indicate that analysis of both mRNA and protein levels would fit into the model of modern diagnostics using complementary molecular markers, allowing early detection of changes and determining the degree of their invasiveness.

In the control material, no expression of studied proteins was observed, whereas in the tumor tissue they were detectable. Thus, the detection of discussed proteins collected in tissue samples would be synonymous with the diagnosis of the neoplastic process, which would be a valuable complement to the histopathological examination. This analysis would also allow to determine and strengthen the certainty that the neoplastic change has been removed with a sufficient margin (no expression of NRP1 and NRP2 in the control material). In our study, we observed that the expression of both proteins increased with cancer grade. NRP-1 expression was found exclusively in tumor cells, whereas the expression of NRP-2 was demonstrated in both vascular endothelium and tumor cells. It is important to emphasize that increasing cell membrane expression of NRP-2 was observed in G1–G3 cancer cells. Elevated expression of both proteins creates ideal environment for tumor development. NRP-2 expression on the surface of endothelial cells helps in the development of vessels, especially lymphatic vessels. There are evidences confirming that NRPs are also mediators of angiogenesis, for example, in cancer [30]. It was also found that they interact

and modulate the function of VEGFR1 and VEGFR2 [31]. Studies suggest that VEGF promotes angiogenesis by acting through NRP-2, while at the same time SEMA3F acts as an inhibitor of angiogenesis. By regulating integrins, VEGF/NRP signaling affects the degree of differentiation of the tumor [26]. We are particularly interested in the emerging concepts of VEGF/NRP signaling in cancer cells, which promotes tumor aggressiveness and that NRP may partially act as a mediator of such action to modulate the function of integrins [32]. Some of the studies have suggested that NRPs mediate the proangiogenic effect by binding to VEGF and antiangiogenic effect through interactions with class 3 semaphorins, which are believed to compete for VEGF binding [33–36]. It has been demonstrated that NRP-2 expression in breast and prostate cancer is correlated with aggressive disease [29]. The observation made by Okon *et al.* indicates that the expression of NRP-1 correlates with metastatic potential of endometrial cancer. In addition, these researchers found the connection between level of NRP-1 and VEGFR2, HGF and C-GSF [37]. In our study, the expression of NRP-1 increased with endometrial cancer grade (G1–G3). It suggests that NRP-1 might be used as diagnostic and therapeutic targets. However, the analysis of NRP-1 expression in ovarian cancer made by Bednarek *et al.* suggest no association between NRP-1 and histological, clinical features of this type of cancer [38]. Taking into account these observations, it seems that the possibility of using NRP-1 as a molecular marker depends on the type of tumor.

As previously mentioned, NRP-2 is involved in many processes related to the development of cancer, and therefore its detection can constitute a diagnostic and prognostic tool in many tumors, including endometrial cancer. Literature data indicate that NRPs expression in malignant tumor cells with poor expression in healthy tissues provides the opportunity to use NRP-2 and NRP-1 as targets for new therapies of endometrial cancer that inhibits or slows the development of tumors. In this context, the results of our study suggest that NRP-1 and especially NRP-2 may be interesting targets for further pharmacological evaluation [29, 31–34].

CONCLUSION

Our research shows that the expression of NRP-1 and NRP-2 increases with the endometrial cancer grade. It seems, therefore, that the increase in expression of NRPs harmonizes with the decrease in the differentiation of tumor cells, and thus its more aggressive nature. The maintenance of overexpression both at the transcript and protein levels indicates an important role of molecular research in modern diagnostics. In addition, molecular biology techniques can complement each other to provide full information about the

expression of the factor of interest and allow a holistic view of the patient.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Approval of the Bioethical Committee of the Medical University of Silesia, no. KNW/0022/KB/237/16 has been obtained for this study. Informed consent was obtained from all of the patients recruited.

HUMAN AND ANIMAL RIGHTS

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

CONSENT FOR PUBLICATION

Informed consent was obtained from all of the patients recruited.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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All authors were responsible for the concept and design of the study, collection and collation of data, analysis and interpretation of data, writing of the article, reviewing, and final reviewing of this article and graphics performance.

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