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Epidermal Growth Factor Receptor (EGFR) Expression in Childhood Brain Tumors

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Abstract. *Overactivation of epidermal growth factor receptor (EGFR) signaling has been recognized as an important step in the pathogenesis and progression of multiple forms of cancer of epithelial origin. Reports regarding EGFR family members in brain tumors are sparse and, thus, the significance of EGFR expression in childhood brain tumors is unclear. In this study, the expression of the EGFR family members was analyzed in 22 medulloblastomas. During the immunohistochemical study, a sensitive, four-step, alkaline phosphatase conjugated antigen detection technique was employed. The results demonstrated the presence of c-erbB-2 (HER-2) and c-erbB-4 (HER-4) in 10 to 50% of the neoplastic cells of high-grade glial tumors with high immunoreactivity, while c-erbB-3 (HER-3) was only detected in less than 10% of the neoplastically-transformed cells. In a follow-up, 70% of children, usually under 4 years of age, with c-erbB-2 (HER-2)-positive MEDs/PNETs, succumbed to the cancer. The Kaplan-Meier estimation revealed a significant correlation between c-erbB-2 expression and survival (p=0.002), suggesting that c-erbB-2 (HER-2) is probably a prognostic marker for limited survival. Medulloblastoma is the most common malignant brain tumor that occurs during childhood. Multimodality treatment regimens have substantially improved survival in this disease; however, the tumor is incurable in about one-third of patients with medulloblastoma, and the current treatment has a detrimental effect on long-term survivors. As such, the results of this study further support the idea that targeting EGFR alone, or in combination with its downstream mediators, represents a promising new approach for the management of childhood brain tumors. Moreover, c-erbB-2 (HER-2) expression may also be of use in better classifying brain tumors.*

"The growth and proliferation of cells are usually tightly regulated processes that are activated by stimuli from their environment. Epidermal growth factor (EGF)-related peptides represent a class of molecules that can trigger cell proliferation, among several cellular processes, such as differentiation, migration, and survival. Binding of EGF-like peptides to the EGF receptor (EGFR) at the cell surface leads to a cascade of intracellular reactions that transduce signals to the nucleus, resulting in particular gene expression patterns. However, in many tumor cells, the regulation of EGFR activity is lost, due to increased or aberrant expression of the receptor or its ligands, and this contributes to many processes important for tumor growth, including cell proliferation, survival, angiogenesis, invasion, and metastasis."
- Janmaat and Giaccone, 2003 (1)

Epidermal growth factor (EGF) and its receptor (EGFR) were originally isolated from the "*nerve growth-promoting substance*" by Cohen in 1960, and the search for tissue-specific factors involved in the regulation of cell growth and division began its dynamic advance (2). The human homolog of EGF was originally named urogastrone, and was found to inhibit gastric acid secretion, demonstrating that growth factors are able to regulate some basic physiological processes (3). The type I family of growth factor receptors, the erbB family of receptor tyrosine kinases, consists of the epidermal growth factor receptor (EGFR), also known as c-erbB-1, c-erbB-2 (HER-2/neu), c-erbB-3 (HER-3) and c-erbB-4 (HER-4) (4-9). The sequence identity between these polypeptides is about 40% to 60% in their

Abbreviations: AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; MoAB, monoclonal antibody; IP, immuno-phenotype.

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extracellular domains and 60% to 80% in their intracellular tyrosine kinase domains (10). The EGFR, a 170 kD transmembrane glycoprotein, contains an intracellular domain with intrinsic protein kinase activity, important in the "vertical" pathway of post-receptor signal transduction (11). It is well known that EGF is a potent mitogen for a number of human tissues (12-14). During the past decade, a number of ligands to EGFR have been identified: EGF, transforming growth factor α (TGF-α), amphiregulin (AR), heparin-binding EGF (HB-EGF), epiregulin and β-cellulin (15-19). The distribution of EGFR in normal human tissues, as detected by immuno-histochemistry, has been demonstrated by Damjanov and co-workers (20).

The human c-erbB-2 oncogene encodes a 185 kD transmembrane protein (p185-HER2) which shares a significant sequence homology with the intracellular portion of the EGFR and the v-erbB oncogene of the avian erythroblastosis virus (21-23). None of the fully characterized ligands bind directly to the c-erbB-2 oncogene protein, but observations of its biology have led to the identification of a family of ligands, the so-called neuregulins (24, 25), which include the neu-differentiation factors (26, 27), heregulins (28), which are ligands with acetylcholine receptor-inducing activity (29), and glial growth factor (30). The EGFR and c-erbB-2 receptors, when expressed at high density, may have cell-transforming properties, suggesting that they can act as dominant oncogenes.

The c-erbB-3 oncogene receptor is expressed in normal human tissues, with a high density on mature and differentiated cells of the gastrointestinal tract and in the neurons of the central nervous system (18). The 6.2 Kb c-erbB-3 specific mRNA has been observed predominantly in epithelial tissues. It is now clear that the members of the NDF/heregulin family of EGF-like molecules bind to the c-erbB-3/HER-3 protein (27-29, 31, 32). Significantly elevated levels of its normal transcript were detected in several breast cancer cell lines *in vitro,* with neither amplification nor gene rearrangement (33).

It is well established that selected growth factors, proto-oncogenes and some chemicals play pivotal roles in normal human intrauterine ontogenesis and are also quite important in embryonal organogenesis, as well as neoplastic transformation (34). EGFRs were found to be intensely expressed in the villous cytotrophoblasts in human placenta during the first trimester (35). The c-erbB-2 (HER-2) oncoprotein has also been detected during the first and third trimesters along the apical membrane of the syncytiotrophoblast, in cells which lacked Ki-67 expression. In advanced pregnancy, EGFR immunoreactivity was localized on the proliferative and differentiated villous trophoblast. In contrast, c-erbB-2 was located only in more differentiated trophoblasts.

Another embryological study in mammals reported that mutant mice embryos carrying a c-erbB-2 null allele died before day 11 of intrauterine ontogenesis, probably as a result of dysfunctions associated with a complete absence of cardiac trabeculae. The development of the neural crest (NC)-derived cranial sensory ganglia and the ontogenesis of motor nerves was also markedly reduced (25). Both c-erbB-2 and neuregulin were detected in NC cells migrating away from the neural tube, suggesting that both molecules act *via* an autocrine mechanism and play a key role in the development and differentiation of NC-related structures. Both molecules were also found in the developing heart; c-erbB-2 was detected in cardiac myocytes, whereas neuregulins were expressed in the adjacent endocardium. These results suggest that both molecules promote heart development *via* a paracrine mechanism (36). During the past two years, neuregulin-deficient, as well as c-erbB-2- and c-erbB-4-deficient mice have been bred genetically and experimentally observed (37,38). Neuregulin-deficient mice display a cranial-neural and cardiac pathology remarkably similar to that of c-erbB-2-deficient mice. The c-erbB-4 deficiency was associated with the lack of cardiac trabeculae development, much like the c-erbB-2 deficiency. In the human thymus, EGF has been shown to promote the acquisition of a neural immunophenotype (IP) by the cells of the reticulo-epithelial (RE) cellular network and also to enhance neuropoietic cytokine secretion (39).

EGFR overexpression has been observed in human neoplasms including breast, ovarian, prostate, bladder, lung, brain and pancreas (40-47). In head and neck squamous cell carcinoma (HNSCC), EGFR expression is reported in approximately 90% of specimens and is associated with a poor prognosis (47,48). EGFR appears to contribute to the growth and survival of neoplastically-transformed cells, in addition to maintaining normal cellular function. The EGFR signal transduction pathways contribute to the development of malignancies through various processes, such as effects on cell cycle progression, inhibition of apoptosis, angiogenesis, tumor cell motility and metastases (49-51). Therefore, many strategies to block or down-regulate EGFR have been developed to inhibit tumor proliferation and improve overall clinical outcome (52). Combining anti-EGFR therapy with chemotherapy or radiation therapy may result in synergistic antitumor activities by inhibiting various processes that contribute to tumor growth (53).

The specific aims of this study were: i) to identify the expression of c-erbB-2 (HER-2), c-erbB-3 (HER-3) and c-erbB-4 (HER-4) in MEDs/PNETs; ii) to determine whether c-erbB-2 (HER-2), c-erbB-3 (HER-3) and c-erbB-4 (HER-4) might be significant diagnostic and prognostic markers for MED/PNET.

Materials and Methods

Tissues and tissue handling. In this immunohistochemical study, formalin-fixed, paraffin wax-embedded tissue sections of human primary childhood anaplastic astrocytomas (ASTRs) and MEDs (DAKO Corporation, Carpinteria, CA, USA) were employed. The diagnoses of the specific subtypes were established according to the WHO guidelines for the classification of glioma by a clinical neurohistopathologist (54-58). Technical details of the immunohistochemical techniques used in this study have already been elaborated by other investigators (59-66) and in the studies previously published by our group (67-72).

Monoclonal antibodies. Anti-c-erbB-2 mouse monoclonal antibody was obtained from Labvision (Fremont, CA 94539, USA; Cat. #MS-441-R7) in a Ready-to-use for Immunohistochemical Staining form. Ab-12 is directed against the cytoplasmic domain of the human c-erbB-2 protein. Isotype: IgG_1. Clone: CB11.

Anti-c-erbB-3 mouse monoclonal antibody was obtained from Labvision (Cat. #MS-725-R7) in a Ready-to-use for Immunohistochemical Staining form. Ab-8's epitope is the extracellular domain. Isotype: $IgG_{1/k}$. Clone: SGP1.

Anti-c-erbB-4 mouse monoclonal antibody was obtained from Labvision (Cat. #MS-637-R7) in a Ready-to-use for Immunohistochemical Staining form. Ab-4's epitope is aa 1249-1264. Isotype: IgG_{2b}. Clone: HFR-1.

Antigen retrieval. No single antigen retrieval solution works well with all antigenic epitopes and, as such, directions from the antibody manufacturer should be followed. In this immunocytochemical research project, the immunohistochemical method of "antigen liberation" or "antigen retrieval" was employed when necessary (73-78). In the first step, antigen retrieval was sometimes achieved by single or combined enzymatic digestion (ficin, pepsin and trypsin from Zymed Labs., South San Francisco, CA, USA) prior to the primary antigen-antibody reaction. Heat-induced epitope retrieval (HIER) (79, 80), as modified by our group, was also employed. Staining of the formalin-fixed and paraffin wax-embedded tissue sections required an antigen retrieval technique, employing preliminary boiling in 10 mM citrate buffer, pH 6.0 (NeoMarkers, Fremont, CA, USA; Cat. # AP-9003) for the anti-c-erbB-2 antibody for 10-20 minutes followed by cooling at room temperature for 20 minutes. Unmasking of fixed antigen epitopes was also carried out by a single or combined enzymatic digestion prior to the primary antigen-antibody reaction. An increase in the quantity of detectable antigenic epitopes following HIER has been described for a number of antibodies. Our method using citrate-buffer solution worked well. Immunomorphological observations reported a significant increase in the intensity of immunostaining in response to this antigen retrieval technique.

Immunohistochemical controls. In order to ensure the specificity of the antibody used in this study, the immunoreactivity of several normal human control tissues were tested including: brain, adrenal, heart, stomach, small intestine, large intestine, liver, kidney, pancreas, lung, testis, ovary, uterus, prostate, thyroid and spleen, all included in one checkerboard multitissue block (DAKO Corporation, Carpinteria, CA, USA; Code # T1065) (81, 82). Several postnatal human thymic specimens were also used as negative and positive tissue controls. A number of neoplastically-transformed tissues, including malignant melanoma and lung cancer tissues, represented the positive tissue controls. Additional controls for all tissues and MoABs included: i) omission of the primary anti-c-erbB-2, anti-c-erbB-3 and anti-c-erbB-4 MoAB; ii) utilization of only the enzymatic developer solution to detect the presence of endogenous alkaline phosphatase activity; and iii) utilization of MOPC 21 mouse myeloma IgG_1 (ICN Biochemicals, Costa Mesa, CA, USA) as a replacement for the primary MoAB to determine non-specific myeloma protein binding to the antigen epitopes of the screened tissues.

Immunohistochemical evaluation. Qualitative and quantitative evaluation of the percent of antigen-positive cells and the intensity of the immunostaining were conducted using a light microscope (Olympus, America, Melville, NY, USA), counting 100-200 cells from each of five to eight distinct areas in non-necrotic, ASTR, melanoma, lung adenocarcinoma and postnatal thymus tissues. Artifacts were avoided, while, on the other hand, morphologically characteristic areas were sought out. The presence of neoplastically-transformed astrocytes and oligodendrocytes with heterogeneous IPs, the endothelial elements of small blood vessels, tumor infiltrating leukocytes and macrophages (the host's immunological effector cells) required careful qualitative assessment. Non-vascular elements were also examined, but only morphologically distinct ASTR cells were scored.

Quantitative evaluation (83): (++++) over 90% of the total cell number are positive; (+++) 50% to 90% of the total cell number are positive; (++) 10% to 50% of the total cell number are positive; (+) 1% to 10% of the total cell number are positive; (±) under 1% of the total cell number are positive; (–) negative.

Qualitative evaluation (83): (A) very intense red staining; (B) strong red staining; (C) light red staining; (D) negative staining.

Results

In our systematic immunohistochemical screening, the expression profiles of c-erbB-2 (HER-2), c-erbB-3 (HER-3) and c-erbB-4 (HER-4) (internal and external domains) were observed in 22 MEDs/PNETs. Eleven out of the MEDs/PNETs demonstrated high levels of immunoreactivity (A,B), detectable by the immunostaining intensity. In 11/22 MEDs/PNETs, 10 to 50% (++) of the tumor cells showed c-erbB-2 (HER-2) and c-erbB-4 (HER-4) positivity, while c-erbB-3 (HER-3) expression was detected in only >10% (+) of the neoplastically-transformed cells.

The expressions of c-erbB-2 (HER-2) and c-erbB-4 (HER-4) were detectable only in high-grade glial tumors, in 9 anaplastic astrocytoms (AAs) and 6 glioblastoma multiforme (GBMs) in 10 to 50% (++) of their tumor cells with identified strong immunoreactivity (A,B immunostaining, stronger in GBMs).

The follow-up on survival rate demonstrated that 70% of patients, revealed to have c-erbB-2 (HER-2)-positive MEDs/PNETs (usually children under 4 years of age), succumbed to their neoplastic disorder. In our opinion, c-erbB-2 positivity is a marker of poor prognosis in MEDs/PNETs. The Kaplan-Meier estimation revealed a

significant correlation between c-erbB-2 expression and survival ($p=0.002$). In further correlative statistical observations, the expression of synaptophysin and glial fibrillary acidic protein (GFAP) in 11/22 MEDs/PNETs revealed a negative correlation between the expression of c-erbB-2 (HER-2) and these two marker proteins. These results suggest that c-erbB-2 (HER-2), which may be predominantly expressed by more embryonal MEDs/PNETs, may delineate a poorer prognostic subgroup, especially in children diagnosed with MED/PNET prior to their fourth year of life.

Discussion

Members of the EGFR family, especially the EGFR and c-erbB-2 (HER-2) receptor, have frequently been implicated as important prognostic indicators in a variety of human malignancies (41, 84-86). The overexpression of any members of the EGFR family suggests the presence of dynamic IP changes in neoplastic cells as a result of continuous and rapid cell transformation. In breast carcinomas (BCs), the overexpression of the c-erbB-2 oncogene protein, combined with the presence or absence of gene amplification, possesses significant prognostic value for the evaluation of decreased survival in the lymph node-positive stage of the disease, but does not correlate well with neoplastic progression (87-94). The epidermal growth factor receptor (EGFR) pathway is also frequently up-regulated in high-grade gliomas such as BCs *via* gene amplification, and by specific mutations that render EGFR constitutively active (95). Amplification of *c-myc*, but not c-erbB-2, is associated with high proliferative capacity in BCs (96). In a similar fashion, overexpression, as evidenced by this study, of EGFR correlates with enhanced malignant potential for childhood brain tumors.

The expression of c-erbB-2 (HER-2) and c-erbB-3 (HER-3) in human malignant melanomas has been described (97). The unregulated expression (overexpression) of the *c-erbB-2* gene, involved in cellular growth, may be mediated by either amplification or post-translational stabilization and has been reported in 25-30% of primary, heterogeneous human BC cells (98-101). The expression of the c-erbB-2 oncoprotein has been associated with poor prognosis and high cellular proliferation activity in a variety of human malignancies, including GBMs (102), astrocytomas (103), lung carcinomas (104), gastric carcinomas (105), bladder carcinomas (106), prostate carcinomas (107, 108), ovarian carcinomas (109), endometrial carcinomas (110-114) and BCs (115-123). In numerous cases, however, no statistically significant correlation between c-erbB-2 expression and the prognostication of disease progression could be established (124-127). Press and co-workers (128) attributed this variability to differences in the tissue processing (fixation), epitope and antigen retrieval methods employed, and the sensitivity of the particular antibody employed in detecting the c-erbB-2 oncoprotein.

The c-erbB-3 protein has been reported to be overexpressed in breast (129), gastrointestinal (130-132) and pancreatic (133) cancers, but no clinical or prognostic importance of this oncoprotein has yet been described. Expression of the c-erbB-3 protein, as our results indicate, is, however, quite limited in brain tumors, which is further evidenced by the fact that no literature exists on c-erbB-3 expression in brain tumors. Cooperative signaling of the c-erbB-2 and c-erbB-3 oncoproteins has been demonstrated *in vitro* (134). Transphosphorylation of c-erbB-3 by c-erbB-2 has been observed in cell lines co-expressing these two oncoproteins, while no such occurrence was detected in cell lines transfected with non-functional c-erbB-2. In addition, the formation of heterodimers of c-erbB-2 and c-erbB-3, with much greater catalytic activity than monomers, has also been established as a way of cooperative signaling by these two oncogene products (134). Thus, the detection of the co-expression of these two oncoproteins may have more clinical and prognostic significance than the detection of overexpression of just the c-erbB-3 protein.

The overexpression and aberrant function of EGFR, c-erbB-1 (HER-1) and its ligands and co-receptors in a wide spectrum of epithelial neoplasms have provided a rationale for targeting this signaling network with novel biological treatment approaches (135,136). The crucial role of EGFR in neoplastic proliferation and the overexpression of EGFR mean that it is often associated with an aggressive IP in brain tumors, such as in over 50% of cases of non-small cell lung cancer(NSCLC), HNSCC and colon cancer. Several EGFR-targeting agents have been recently developed (C225, ABX-EGF, E7.6.3, EMD 55900, ICR62, ZD1839, CP358774, PD168393, CGP75166/PKI166, CGP59326A, BIBX1382). The two most advanced EGFR inhibitors in development are C225 and ZD1839. C225 is an antibody directed against the ligand-binding domain of human EGFR, which competes for receptor binding with EGF and other ligands (137). *In vitro*, C225 inhibits EGFR tyrosine kinase activity and proliferation of EGFR-overexpressing squamous cell carcinoma cell lines. Synergy was observed with doxorubicin, cisplatin and radiation in preclinical animal models.

Encouraging data on two new, so-called "targeted drugs", that basically jam up the cancer's internal signaling circuits without producing major side-effects, have been reported (138). OSI Pharmaceuticals' Tarceva was found to be robust both as monotherapy and in combination with chemotherapies, because it resulted in significantly less tumor growth and, in some cases, even partial regressions (139). ImClone system's Erbitux had similar results (138).

Several other anti-receptor therapeutic strategies have been pursued, with two standing out ahead in their clinical development. One approach has been the generation of small

molecules that compete with adenosine triphosphate (ATP) for binding to the receptor's kinase pocket, thus blocking receptor activation and the transduction of postreceptor signals. The second approach utilizes humanized monoclonal antibodies (MoABs) generated against the receptor's ligand-binding extracellular domain. These antibodies block binding of receptor-activating ligands and, in some cases, can induce receptor endocytosis and down-regulation. The antibodies may act directly when binding to a target molecule by inducing apoptosis, inhibiting cell growth, mimicking or blocking a ligand, or interfering with a key function (139, 140). Moreover, those antibodies may also modulate or potentiate drug effectiveness or other therapies. The antibody may itself act as an effector by inducing antibody-dependent cellular cytotoxicity (ADCC) or an antibody-dependent complement-mediated cascade, or it may involve effector elements such as cytotoxins, enzymes, radioactive isotopes, signals for other parts of the immune system, and/or cytotoxic drugs (141-145). Antibodies can also be conjugated to cytotoxic drugs, radioisotopes, or toxins (142, 146-148) and, as such, MoAbs have been conjugated to chemotherapeutic drugs such as doxorubicin, cisplatin, carboplatin, mitomycin and methotrexate. Clinical studies already suggest that both of these approaches, either alone or in combination with standard anti-neoplastic therapies, are well-tolerated and can induce favorable clinical responses and tumor disease stabilization in a variety of common solid neoplasms.

Herceptin is the most well known antibody, because of its ability to be used in such a wide spectrum. The oncoprotein Her-2/neu is a growth factor receptor with tyrosine kinase activity implicated in the process of neoplastic progression. It has both prognostic and probably predictive significance. HER-2 gene amplification/protein overexpression is associated with more aggressive neoplastic disease progression and immunophenotypical changes in the embryonal direction, which thereby shortens survival (87, 149, 150) by altering responses to conventional anti-neoplastic therapeutic modalities (151, 152).

Trastuzumab (Herceptin) is a humanized MoAb, that binds directly to the cell surface HER-2 receptor c-erB-2 protein (HER-2). It has recently been approved by the FDA for the treatment of breast cancer (153-158). The antibody was developed by humanizing the murine MoAb 4d5 by inserting antigen-binding regions of MoAb 4d5 into the framework of a consensus human IgG1, resulting in rhuMabHer2 or trastuzumab (154). In preclinical studies, it was found that Herceptin exerted a cytostatic effect on BC cells overexpressing HER-2, down-regulated the number of HER-2 receptors on the cell surface and supported antibody-dependent cellular cytotoxicity (ADCC) against established human tumor cell lines. Herceptin, however, had no impact on healthy HER-2-negative cells (159, 160), with synergistic and additive cytotoxic effects being observed when Herceptin was combined with chemotherapeutic agents including cisplatin, paclitaxel and doxorubicin (161-163). Moreover, withdrawal of Herceptin treatment in preclinical models resulted in rapid neoplasm regrowth (163).

Herceptin has been employed as monotherapy or in combination with chemotherapy in $HER2^+$ metastatic breast cancer patients (164-166). Two major phase II trials of Herceptin as monotherapy have been conducted, both of which demonstrated that it had significant clinical benefit (164, 165). When used as second- or third-line monotherapy in heavily-pretreated $HER2^+$ women with stage IV breast cancer, Herceptin produced an overall response rate of 15%, a median duration of response of 9.1 months and survival of 13 months (164). The observed benefit was even greater when Herceptin was used as first-line monotherapy, with the overall response rate increasing to 26% (165).

The side-effects associated with Herceptin are generally mild to moderate in severity. The most significant event associated with treatment in these trials was cardiotoxicity (164, 165), but it should be duly noted that the majority of these patients had underlying cardiac disease and prior anthracycline exposure. Overall, most patients achieved benefit from Herceptin therapy (167). The clinical trials reported to date have demonstrated that the addition of Herceptin to paclitaxel, anthracycline/cyclophosphamide (AC), docetaxel and vinorelbine produced clinical benefit greater than that of chemotherapy alone (166, 168). In fact, when strongly $HER2^+$ patients were evaluated after 35 months of follow-up, the overall survival was shown to increase by 45% from 20 months in patients treated with chemotherapy to 29 months in those treated with Herceptin plus chemotherapy (169).

While existing data provide clear support for the use of Herceptin until disease progression, treatment beyond progression could also be beneficial. In a phase III trial, patients continued to receive Herceptin after disease progression (170). The response rate among these patients was 11% and response duration was 6.7 months. Furthermore, no new side-effects of Herceptin were observed with up to 12 months of therapy, indicating that long-term exposure is well-tolerated by the body.

These data indicate the potential for MoAb therapy directed against EGFR signaling. Several molecular strategies have been developed recently to modulate either EGFR or the downstream signal beyond the cell surface receptor. The important role of aberrant EGFR signaling in the progression of malignant gliomas makes EGFR-targeted therapies of particular interest for brain tumors. The use of anti-EGFR therapies against malignant brain tumors, although in its infancy, promises to yield exciting results like that of Herceptin for BC, as these new drugs will probably enhance the usefulness of existing classical modality therapies.

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