Antisense epidermal growth factor receptor (EGFR) transfection down-regulates EGFR ex鄄pression and suppresses the malignant phenotype of human nasopharyngeal carcinoma CNE-2 cell line

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Abstract: Objective Todeterminewhetherepidermalgrowthfactorreceptor(EGFR)expressioncontributestotumorgrowth of poorlydifferentiatedhumannasopharyngeal cacinoma CNE-2cell lines. **Methods** An expression vector containing a N-terminalfragment(1.35kb)ofhumanEGFRintheantisenseorientationwastransfectedintoCNE-2celllinesvialipofectamine.Theestablishedclonesresistantto G418wereisolatedandcharacterized,andthetumor-inhibitingeffectofantisense EGFRexpressionwasevaluatedintermsoftumorgrowthandmetastasisatdifferenttimeaftersubcutanenousinoculationinto nudemice. **Results** Down-regulatedEGFRexpressioninthecellswithantisensevectortransfectionwasdemonstratedbyligandbindingassay. Thegrowthrateandtheabilitytogrowinsoftagaroseoftheseantisensetransfectantswerealsoreduced. Afterinoculationintonudemice,EGFRantisensetransfectantsshowedalongerlatencyperiod,slowertumorgrowthandlowermetastaticratestothelymphnodesandlungincomparisonwiththeparentalcells. **Conclusions** Theseresultssuggestthat these EGFRantisensecDNA-transfectedCNE-2cellsareofvaluetofurtherdelineatetheroleofEGFRinthedevelopment andprogressionofnasopharyngealcarcinoma.

Key words: epidermalgrowthfactorreceptor;humannasopharyngealcarcinomacells;antisenseRNA;genetransfer

Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein with relative molecular weight of 17 000, consisting of extracellular ligand bindingdomain, transmembranedomainandintracellular tyrosine-kinasedomain. Alargenumberofligands forEGFR have been characterized, includingepidermal growthfactor(EGF), transforminggrowthfactor琢TGF琢 andamphiregulin. Bindingoftheseligandstoextracellular domainofEGFRinitiatesavarietyofbiologicalresponses, resultingintheactivationofmitogenic signal transduction pathway to maintain balanced proliferation[1,2].

Several investigations have shown that amplificationand/oroverexpression ofEGFR gene maybeassociated with the growth rateandmalignancy of the tumors, whichinturnhasprognosticssignificance inpatients with these tumors[3], suggesting that receptor-directedtherapies may be useful asanticancer strategies. Although EGFR down-regulation by antisense EGFR cDNA transfection has been demonstrated in human breast cancer[4,5], rhabdomyosarcoma[6], colon cancer[7], non-small-cell lung cancer [8], and glioma cells[9], no suchefforts havebeenattemptedinhumannasopharyngealcarcinomacells.

Nasopharyngeal carcinoma (NPC) is one of the most frequent cancers in south China. Although Epstein-Barr virushasbeenshowntobethelikelyetiologicagentonnasopharyngealcarcinogenesis, the molecular pathogenesis ofNPC remains tobeelucidated. The firstinsightintothepotentialroleofEGFRinNPCprogression came from the observations of a monoclonal antibody against EGFR, which significantly inhibited the growth andtumorigenesisofhumanNPCcellsboth **in vitro** and **in vivo** [10]. Inthisstudy, wetransfecteda plasmid containing a 1.35-kb gene fragment encoding the N-terminal fragment of humanEGFRcDNAinthe 3' to 5' orientation intoNPCCNE-2celllinewithhigh EGFR expression and examined the effects of down-regulatedEGFRexpressiononthe growthbehaviorandtumorigenesisofthecellsinnudemice.

MATERIAL AND METHODS

Cell lines and plasmids

HumannasopharyngealcarcinomaCNE-2cells [11] were cultured in RPMI 1640 medium supplemented with10%fetalbovineserum(GIBCO/BRL).A1.35-kb BamH1fragmentcontainingtheextracellularregionfor EGFR derived from a full-length 4.0-kb human cDNA was cloned in the reverse 3' to 5' orientation into the pLXSN retrovirusvector aswedescribedpreviously [10].

Plasmid transfection and production of stable cell lines

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The antisense EGFR expression vector pLXSN-AS5andpLXSNcontrolvectorwererespectivelytransfected into CNE-2 cells via lipofectamine (GIBCO/BRL) accordingtothemanufacturer'sinstructions. Several G418-resistant clones were subsequently isolated andexpanded. Thestableintegrationandstructuralintegrityoftheplasmid DNA wereexamined byPCRas wedescribedpreviously[噎], andtheselectedclonesofthe cells with antisense EGFR expression vector transfection were designated as CNE-2/AS4 and CNE-2/AS8 respectively; pLXSNvector-transfectedcellsweredesignated asCNE-2/pLXSN.

^{125}I-EGF ligand binding assay

This assay was performed as describedpreviously[噎]. The parental cells or transfected cells (CNE-2/pLXSN,CNE-2/AS4, and CNE-2/AS8) were platedat2伊10^5/ well into 24-well Nunc dishes and allowed togrow overnight. The confluent cultures were placed the followingdayoniceandwashedoncewith wash buffer (containing 0.01% Mg^{2+} and0.005%Ca^{2+} plus 1 mg/mlBSAofPBS)pre-cooled to 4 益, prior to theadditionof1mlunlabeledcoldEGF(2.5-500ng/ml) in washbuffer into the triplicate dishesandof ^{125}I-EGF (9伊10^4 cpm) into each well. Theculture dishes were then rocked gently onice for 4 h, the cells washed 5 timeswiththewashbuffer,lysedwithRRAlysisbuffer (HEPESpH7.3, 20mmol/L, 10%glycerol, 1%TritonX-100) and counted in gamma counter. The results wereunderwentcomputer programLIGANDanalysis.

In virto cell growth assay

Theparentalcellsortransfected cells wereseeded at1伊10^4 cells/wellandgrownintriplicate35-mmdishes for8d.Thecellswereharvestedandcountedbytrypan blueexclusiononday0,2,4,6,and8,respectively.

Anchorage-independent growthassay

Softagarose assay wasperformedas describedpreviously[噎]:Inbrief, 1伊10^3 cells/wellwereplatedon0.35% low-melting-point agarose in RPMI 1640 containing 15% fetalbovineserum. Thecoloniesweresco redunderamagnifyingglassafter10days. Eachsoftagarose assay wasdoneintriplicate.

Tumorigenicity and metastasis in nude mice

Theparentalcells ortransfectedcells were injected subcutaneously (1伊10^6 cells) into 6 female nude mice (4-5weeksold).Micewereexaminedtwiceaweekand the tumor size was measured using a caliper. Tumor volumes(mm3)werecalculatedaccordingtotheformula: V=length伊width)2/2[噎]. After 77 days, the mice were killedandthe tumor, lungand lymphnodewere removedrespectively, fixedinneutralbufferformalin, embedded in paraffin, and sectioned for histological analysis.

RESULTS

Effect of EGFR antisense RNA expression on EGFR expression levels and binding affinity

After transfection of CNE-2 cells with the EGFR antisense RNA expression vector or the empty vector, G418-resistant cloneswereidentifiedandisolated. GenomicDNAwasextractedfromthe4selectedCNE-2/AS clones,1CNE-2/pLXSNcloneandtheparental CNE-2 cells.A433-bpfragmentofintegratedneogenesequence wasdetectedinthetransfectedCNE-2cells, but notin the parental CNE-2 cells by PCR analysis (data not shown). Tworepresentative CNE-2 clones CNE-2/AS4 and CNE-2/AS8 were reserved for later experimentation.

^{125}I-EGF binding and Scatchard analysis showed thatthenumberofEGFRwasreducedby18%and45% inthetwoselectedclones(CNE-2/AS4, $P<0.05$;CNE-2/AS8, $P<0.01$)respectively,whereastheclonestransfectedwith the empty vector (CNE-2/pLXSN) had similar EGFR levels to those of the parental cells ($P>0.05$). However, the binding affinity of EGFR to the ligand was not significantly affected byEGFRantisense RNA expression(Tab1).

Tab.1 Binding of ^{125}I-EGF to the parental and transfected CNE-2 clones (Mean依SD)

Cellline	ReceptorNo./cell	Bindingaffinity(Kd,nM)
CNE-2	67658依700	2.50依0.29
CNE-2/pLXSN	62273依900*	2.39依0.18*
CNE-2/AS4	55790依620**	2.13依0.50*
CNE-2/AS8	37512依430***	2.07依0.47*

*$P>0.05$,**$P<0.05$,***$P<0.01$.Thebindingsitesandbinding affinity(liganddissociationconstant,Kd) weredeterminedby Scatchardanalyses.Student's t testwasusedforcomparison betweentransfectedclonesandtheparentalcells.

Effect of EGFR antisense RNA on cell proliferation

In order to examine whether anti-sense EGFR RNAsuppresses the growthofthetumorcells in vitro, we plated 1伊10^4 CNE-2 cells in 35-mm plastic dishes containing RPMI 1640 supplemented with 10% FBS.

Thenumberofviablecellsineachdishwascountedeveryother day. AsshowninFig.1, the proliferationrate significantly decreased in the transfected clones of CNE-2cells expressingEGFRantisenseRNA, ascomparedwiththatoftheparentalcellsorCNE-2cellstransfected withtheemptyvector. A reductionby33%and 55% in the cell number occurred 8 d after the plating of the CNE-2/AS4 and CNE-2/AS8 cells respectively (Fig.1).Theseresultssuggestedthattheextentofgrowth inhibitioninEGFR antisenseRNA-tranfected clones as compared with the parental cells was in proportion to thedegreeofEGFRexpressionthatwasblocked.

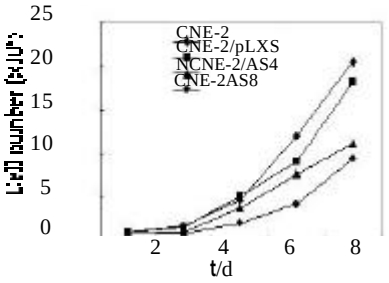

Fig.1 In vitro growth inhibition of CNE-2 cells transfected with EGFR antisense cDNA vector compared with the parental cells or the cells transfected with the empty vector

Effect of EGFR antisense RNA on CNE-2 cell growth on soft agarose

Theparentalandtransfectedcloneswereexamined fortheircolony-formingabilityinsoftagarosemedium, which was an accepted criterion for transformation capacityassessment. As shown in Fig.2, the growth of CNE-2/AS4orCNE-2/AS8cloneswasinhibitedby40% -50%incomparisonwiththeparentalandCNE-2/pLXSN controlclones. Inaddition, asexpected, CNE-2/AS4 andCNE-2Z/AS8cellsformedsmallerandlesscolonies on soft agarose than the parental and empty vector-transfected cells (datanotshown). Theseresultssuggest that EGFR antisense RNAcansuppress **in vitro** the tumorigenicityofCNE-2cells.

Tumorigenicity and metas鄄tasis in nude mice

The tumorigenicity of the parental and transfected cloneswas examinedin6female nude mice by subcutaneousinjection. Asshownin

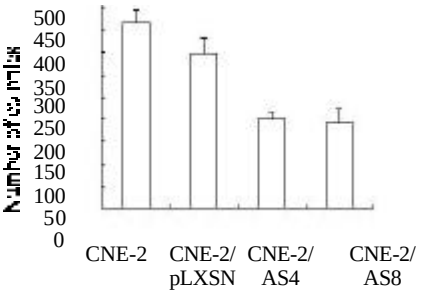

Fig.2 Growth inhibition of CNE-2/AS4 and CNE-2/ AS8 cells in soft agarose

Tab.2, all mice injected with CNE-2 cells and CNE-2/pLXSN cells rapidly developed aggressively growing tumor nodules at the site of inoculation after 3-5 d. However, EGFR antisense RNA-expressing clones showed a longer latency period (9-11 d) and slower tumor growth rate compared with those of the parentalcells and CNE-2/pLXSN clones 1 month after theinoculation(Fig.3).

To investigate the effect of antisense EGFR RNA on the metastasis ofthetumors **in vivo**, allmicewere killedforhistopathologicalexaminationonday77after injection,andthelungsandlymphnodeswereremoved. The sections obtained for histological analysis showed that CNE-2/AS4cellshadnolungmetastasiswithlower rate of lymph node metastasis than the parental and CNE-2/pLXSNcells, while CNE-2/AS8 cells had significantlydifferent lymph node metastasis from that of theparentalcells(Tab.2).

DISCUSSION

Overexpression of the epidermal growth factor receptor is frequent inhumancarcinoma, often accompanied bythe expression of autocrine or paracrine transforminggrowthfactor 琢 which resultsintheactivation ofEGFRtyrosine kinase and phenotype transformation [1,2]. CNE-2 cell line wasestablished from biopsy of a poorly differentiated and moderately metastatic nasopharyngeal carcinoma[3].Previousinvestigationshave demonstratedthatEGFRoverexpressionassociateswith

Tab.2 Tumorigenicity and metastasis analysis of CNE-2 cell line transfected with antisense EGFR cDNA

Cellline	Nudemice	Tumorformation(days afterinoculation)	Lungmetastatsis/total lunglobes	Lymphnodemetastasis/ totalnumberoflymphnodes
CNE-2	6	3-5	4/25(16%)	28/39(71.8%)
CNE-2/pLXSN	6	4-5	3/25(12%)	22/44(50.0%)
CNE-2/AS4	6	9-11	0/30(0%)	19/43(44.2%)
CNE-2/AS8	6	10-11	2/30(6.6%)	24/42(57.1%)

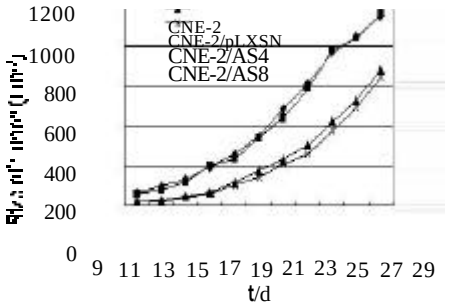

Fig. 3 Inhibitory effect of EGFR antisense RNA expression on tumor growth in nude mice

thegrowthandtumorigenesis ofCNE-2, which canbe significantly inhibited by a monocolonal antibody a gainst EGFR[咱暫]; andweconsequentlychosethiscellline tostudytheeffectofEGFRantisenseRNAexpression.

Previous therapeutic strategies designed to interferewithEGFRsignaltransductionhave used antisense oligonucleotides[咱暫]; antisense RNA[咱6,9,8暫]or the antibodiesagainstEGFR[咱7-19暫].Inthisstudy,byanantisenseRNA approach,weestablishedCNE-2/AScells(CNE-2/AS4, CNE-2/AS8) inwhichantisenseRNAwasgeneratedby a sequencethatwasstablyincorporatedintothegenome for consistent expressionofthetranscript. EGFR antisense RNA expression could significantly down-regulate the number of EGFR on the surface of the two CNE-2 cells transfected with EGFR antisense cDNA vector,ascomparedwiththeparentalandCNE-2/pLXSN cells. However, the binding affinityof theEGFRforlig andisnotsignificantlyaffectedbyantisenseEGFRRNA. These results are essentiallyinaccordancewiththepreviousfindingofChakrabarty **et al**[咱暫], whodemonstrated that a 31% reduction in the number ofhigh-affinitycell surface EGFR and a 68% reduction in low-affinity cell-surface EGFR occurred in the human colon carcinoma MosercellstransfectedwithEGFRantisensecDNAvector, butthebindingaffinityoftheEGFRforligandremianedunchanged.

The growthrate **in vitro** andtheabilityto grow in soft agarose of these two EGFR antisense vector-transfected cells were also significantlyreduced. Afterinjection into nude mice, EGFR antisense transfectants showed a longer latency period, slower tumor growth andlowerlymphnodeandlungmetastasisratesincomparison with the parental cells. In addition, tumor growth and tumorigenesis were proportional to the degree of EGFR expression inhibitionin cells transfected withEGFR antisensecDNA vectors. However, complete inhibitionofthecellgrowthandtumorigenesiseither **in vitro** or **in vivo** wasnotobserved. Asimilarresultwasobtainedby Moronietal[咱暫], whodemonstrated thattheinhibitionofthephenotypewasdirectlyproportionaltotheresidualamountofEGFRexpressedin humanepidermoidcarcinomaKBcellwithtransfectionof EGFRantisense RNAconstructs.

Antisense RNAstrategyhave been successfullyutilized to reduce the expression of a number of mammaliangenes, includingthoseforgrowthfactors or relatedreceptorgenes[咱0,2,21暫]. This is the firstreporttoaddresstheeffectofEGFRantisense plasmid transfection in inhibiting the tumoregenicity and metastasis of human NPCcells, therebyprovidinga valuable targetfor therapeutic intervention of tumors with EGFR overexpression.

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REFERENCES

咱暫 KhazaieK.EGFRinneoplasiaandmetastasis[咱暫]CancerMetastasis Rev,1993,12(3-4):255-74.

咱暫 MishimaK,AsaiA,SugiyamaA.Increasedexpressionofschwannoma-derivedgrowthfactor(SDGF) mRNAinrattumorcells:involvementofSDGFinthegrowthpromotionofratgliomas[咱暫]Int J Cancer,1996,66(3):352-7.

咱暫 GullickWJ. Prevalenceofaberrantexpression of theepidermal growthfactorreceptorinhumancancers[咱暫]BrMedBull,1991,47 (1):87-98.

咱暫 FanWH,LuYL,DengF, **et al**.EGFRantisenseRNAblocksexpressionoftheepidermalgrowthfactorreceptorandpartiallyreversethe malignant phenotypeofhumanbreastcancerMDA-MB-231cells [咱暫]CellResearch,1998,8(1):63-71.

咱暫 DixitM,YangJL,PoirierMC, **et al**. Abrogationofcisplatin-induced programmedcelldeathin human breast cancercellsbyepidermal growthfactorantisenseRNA[咱暫] J Nat CancerInst,1997,89(5): 365-73.

咱暫 DeGiovanniC,LanduzziL,FrabettiF, **et al**. Antisenseepidermal growthfactorreceptor transfectionimpairstheproliferativeability ofhumanrhabdomyosarcomacells[咱暫] CancerRes,1996,56(17): 3898-901.

咱暫 ChakrabartyS, RajagopalS,HuangS.Expressionofantisenseepidermal growthfactor receptorRNAdownmodulatesthemalignant behaviorofhumancoloncancercells[咱暫]ClinExpMetastasis,1995, 13(3):191-5.

咱暫 FangK,ChenMH.Transfectionofanti-sensecomplementaryDNA

ofhumanepidermalgrowthfactorreceptorattenuatestheproliferationofhumannonsmallcelllungcancercells 咱暂 IntJCancer, 1999,81(3):471-8.

咱暂 TianXX,PangJC,ZhengJ, et al. Antisenseepidermalgrowthfactor receptorRNAtransfectioninhumanglioblastomacellsdown-regulatestelomeraseactivityandtelomerelength咱暂BrJCancer,2002, 86(8):1328-32.

咱10暂GuanEN,ZhouTC,WangJH, et al.Effectsofmonoclonalantibody anti-EGFreceptoronhumannasopharyngealcarcinomacellandother cells咱暂 SciChinaB(Chin),1990,33(11):1334-40.

咱11暂TangWP,HuangPG,ShenSJ, et al.Establishmentandcharacteristicsoftransplantationmodelofhumanepithelialcellstrainofpoorlydifferentiatednasopharyngealcarcinoma咱暂Cancer(Chin.)1989, 8(4):247-50.

咱12暂LichtnerRB, KaufmannAM, KittmannA, et al. Ligandmediated activationof ectopic EGF receptor promotesmatrixprotein adhesionandlungcolonizationofratmammaryadenocarcinomacells 咱暂Oncogene,1995,10(9):1823-32.

咱13暂ArteagaCL,HoltJT.Tissue-targetedantisensec-fosretroviralvectorinhibitsestablishedbreastcancerxenograftsinnudemice 咱暂 CancerRes,1996,56(5):1098-1103.

咱14暂DiFiorePP,PierceJH,HazanR, et al. Over-expressionofthehuman epidermal-growth-factor receptor confers a EGF-dependent transformedphase-typetoNIH3T3cells咱暂Cell,1987,51(6):1063-70.

咱15暂WangS,LeeRJ,CauchonG, et al. Deliveryofantisenseoligodeoxyribonucleotidesagainstthehumanepidermalgrowthfactor Receptor intoCulturedKBcellswith liposomes conjugatedtofolate viapolyethyleneGlycol咱暂 Proc NatAcadSci(Wash.), 1995,92 (8):3318-22.

咱16暂PuP,LiuX,LiuA, et al.Inhibitoryeffectofantisenseepidermal growthfactor receptorRNAontheproliferationofratC6glioma cells in vitro and in vivo咱暂Neurosurg,2000,92(1):132-9.

咱17暂MaL, Gauville C, BerthoisY, et al. CalvoF. Role ofepidermal growthfactorreceptorintumorprogressionintransformedhuman mammaryepithelialcells咱暂IntJcancer,1998,78(1):112-9.

咱18暂DivgiCR,WeltS,KrisM, et al.Phase 玉 andimagingtrialofindium 111-labeled anti-epidermal growth factorreceptor monoclonal antibody225inpatientswithsquamouscelllungcarcinoma 咱暂 J NatlCancerInst,1991,83(2):97-104.

咱19暂BaselgaJ, NortonL, MasuiH, et al. Antitumoreffectsofdoxorubicin in combination with anti-epidermal growth factor receptor monoclonal antibodies咱暂J NatlCancerInst,1993,85(16):1327-33.

咱20暂JenoH,SasakiK,MiyagawaK, et al. HiraiH.Antisenserepression of proto-oncogene c-Cbl enhances activation of the JAK-STAT pathwaybutnottheraspathwayinepidermalgrowthfactorreceptor signaling咱暂J BiolChem,1997,272(13):8739-43.

咱21暂HeY,ZengQ,DrenningSD, et al. Inhibitionofhumansquamous cellcarcinomagrowth in vivo byepidermalgrowthfactorreceptor antisenseRNAtranscribedfromtheU6promoter咱暂J NatlCancer Inst,1998, 90(14):1080-7.

EGFR反义 RNA 的转染对人类鼻咽癌 CNE-2 细胞 EGFR 的表达下调及恶性表型的抑制

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摘要：目的 利用反义 RNA 抑制靶基因表达的策略，下调 EGFR 的表达而探讨对人类低分化鼻咽癌 CNE-2 细胞的恶性表型的抑制性效应遥方法 将人 EGFR 的 N-端 1.35kb 片段反向构建入逆转录病毒表达载体 pLXSN 中，并用脂质体介导转染人鼻咽癌 CNE-2 细胞，G418 筛选并分离阳性克隆，将两个 EGFR 反义 cDNA 转染的克隆细胞命名为 CNE-2/AS4 和 CNE-2/AS8，而空载体转染的细胞命名为 CNE-2/pLXSN遥125I-EGF配体结合分析细胞膜上 EGFR 的表达，台盼蓝染色法测定细胞生长的改变，并用软琼脂集落形成实验检测细胞转化，最后，将筛选的各组阳性克隆细胞分别注射入裸鼠皮下，不同时间观察肿瘤生长的抑制改变及肿瘤的转移状况遥结果 配体结合实验结果显示，两个选择的克隆 CNE-2/AS4 和 CNE-2/AS8 细胞表面 EGFR 的数量分别较未转染细胞 CNE-2 下降 18%和 5%，表明 EGFR 反义 RNA 的表达下调了细胞膜上 EGFR 的表达曰同时，EGFR 反义 RNA 表达的 CNE-2 细胞生长速率和软琼脂生长能力也较对照组细胞明显降低遥注射入裸鼠皮下后，EGFR 反义 RNA 表达的阳性克隆细胞表现出肿瘤生长减慢，淋巴结和肺转移能力也明显降低遥结论 这些实验结果提示 EGFR 反义 cDNA 转染的 CNE-2 细胞能下调 EGFR 的过量表达并部分抑制鼻咽癌的恶性表型，这为进一步阐明 EGFR 在鼻咽癌的发生尧演化中的功能作用提供了有用的工具遥

关键词：表皮生长因子受体 曰人鼻咽癌细胞 曰反义 RNA 曰基因转移