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STUDIES ON THE GAP JUNCTIONAL INTERCELLULAR COMMUNICATION OF HUMAN NASOPHARYNGEAL CARCINOMA CELLS AND THE EFFECT OF RII

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Human nasopharyngeal carcinoma (NPC) cell line, CNE-2Z, and its clones (L_2, H_2, L_4) with various invasive and metastatic potentials were examined for their gap junctions (GJ), gap junctional intercellular communication (GJIC) and the concentration of cytosolic free calcium ($[Ca^{2+}]_i$). Only a few intermediate junction (IJ) but no GJ structures were observed under electron microscope (EM). CNE-2Z cells showed marked JGIC, while its variants lacked this function using the scrape-loading dye-transfer technique (SLDT). There was lower concentration of $[Ca^{2+}]_i$ in L_2 cells (a variant with high invasive and metastatic potential) compared to that in H_2 and L_4 cells (variants with medium and low invasive and metastatic potentials, respectively). These data suggested that high invasive and metastatic potentials might be correlated with the level of $[Ca^{2+}]_i$ in NPC cells.

The effect of RII (4-hydroxycarbophenyl retinamide) on NPC cells also investigated,. After 3–7 d of RII (10^{-5} M) treatment, there was no change in the number of gap junctions and other kind of intercellular junctions in NPC cells observed under EM. The JGIC of CNE-2Z weaked and then disappeared finally with prolonging of RII treatment. However, there was no influence on its variants. The level of $[Ca^{2+}]_i$ in NPC cells apparently fell after 6 h of RII treatment, and rose to original level with persisting of RII treatment. Whether the fluctuating of $[Ca^{2+}]_i$ level is related to the inhibitory effect of RII treatment on growth and invasion of NPC cells needs to be further studied.

Key words: Gap junctional intercellular communication (JGIC), Retinoids, Intercellular free calcium, Invasion, Metastasis.

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Gap junctions, as a specialized membrane contacts between adjacent mammalian cells, provide a path not only for intercellular electrical communication but also for metabolic cooperation. Intercellular communication through gap junctions is considered important in the progression of tumor cells.[1] Evidence suggests that the invasive behavior of tumor cells is correlated with their gap junctional coupling.[2] In general, malignant cell lines have been reported to be communication deficent,[3] there are still datas that gap junctional communication exist in some tumor cells.[4] Intercellular calcium, adenosine triphosphate (ATP), cyclic adenosine monophosphate (cAMP) play a significant part in regulation of gap junctional communication.[5] To explore the relationship between cell communication and malignancy of tumors, we examined the GJIC and the level of intracellular calcium in a human nasopharyngeal carcinoma parent cell line and its variants with various potentials for invasion and metastasis to lymphnode and lung *in vivo* of nude mice. Meanwhile the effect of 4-hydroxy-

carbophenyl retinamide was detected.

MATERIALS AND METHODS

Cells

Human nasopharyngeal carcinoma cell line CNE-2Z was kindly provided by Ji Nan Medical University in Guang Zhou. Cell clones were established from the cell cultures *in vitro* by limited diluting cell suspension. The parent cell line and its various clones were quite different in their abilities to produce invasion and spontaneous lymphnode or lung metastasis in nude mice. CNE-2Z revealed mediate invasion and metastatic potentials. Its clones L_2, H_2, L_4 were high, mediate and low invasive and metastatic potentials, respectively. Tumor cells were grown in RPMI -1640 medium containing 10% fetal serum and antibiotics. Cells were passaged in tissue culture flasks by treatment with 0.25% trypsine and 0.02% EDTA in PBS without calcium and magnesium.

RII Treatment

4-hydroxycarbophenyl retinamide (RII) was obtained from the Department of Organic Synthesis, Institute of Materia Medica, Chinese Academy of Medical Sciences. RII was dissolved in absolute ethanol. Immediately prior to each experiment, RII was added into the cell culture media to 0.0001/L with a final ethanol concentration of 0.1% (v/v). Freshly diluted RII was supplemented to the cell cultures every 72 h. All work with RII was conducted in sundued light.

Electron Microscopy

For transmission electron microscopy, NPC cells were grown on plastic slices in dishes. Monolayer cultured cells were washed in PBS and fixed with 1% glutaraldehyde and 1% polyformol for 2 h. After rinsing with cacodylate buffer, cells were postfixed with 1% osmium tetroxide for 1 h, dehydrated in progressive concentrations of ethanol and finally embedded in Epon 812 resin. This sections were prepared and stained with uranyl acetate and lead citrate for electron microscopic examination with a JEM-2000 EX electron microscopy.

Intercellular Communication

We measured intercellular junctional communication in NPC cells *in vitro* by the scrape-loading dye-transfer technique.[7] Lucifer yellow (LY, Mr 457.2) does not diffuse through intact cell membranes, but its relative molecular mass permits diffusion through patent gap junctions. Rhodamine dextran (RD, Mr 10,000) is used as control dye, because it cannot diffuse through intact cell membranes or gap junction. Cells grown on the glass slices were rinsed with PBS. 0.05% LY and RD (purchased from Sigma) dissolved in PBS were added to the cells and scrape-loaded using a edge of knife. The dye solution was left on the cells for 3 min, then discarded and the glass slices rinsed with PBS. The cells were examined under Opton epifluorescent microscope.

Measurement of Intercellular Calcium

Procedures for spectra of intercellular Fura-2 have been previously described.[8] After NPC cells were loaded with Fura-2, the fluorescence spectra were obtained in a Hitachi 850 scanning spectrofluorometer. Then the value of intercellular calcium were calculated by the formula as follow:

$$[Ca^{2+}]_i = 224\ nm\ (F-Fmin)/(Fmax-F)$$

RESULTS

Morphology

Ultrastructural study revealed that CNE-2Z and its variants had cell junctions in normal monolayer cell culture *in vitro* that appeared to be intermediate junctions, however, no obvious gap junctions were found (Figure 1). After 3–7 d of RII treatment, there was no change in the number of gap junctions and other kind of intercellular junctions observed.

GJIC in NPC Cells

It was found that the parent cell line CNE-2Z showed a marked gap junctional intercellular communication by the dye-transfer assay. The positive transmission of the Lucifer yellow occurred in 3 rows of the contiguous cells at the edge of the scraped areas

(Figure 2). In contract, the clones of CNE-2Z lacked GJIC, Lucifer yellow remained in the cells scraped (Figure 3). The GJIC function of CNE-2Z weakened and then disappeared finally with the prolong treatment of RII (Figure 4–6). However there was no influence on its variants (Table 1).

Table 1. Effects of RII on GJIC of NPC cells

Drugs	Duration of drug treatment	GJIC of cells			
		CNE-2Z	L_2	H_2	L_4
Negative control		+++	–	–	–
RII	6 h	++	–	–	–
RII	3 d	+	–	–	–
RII	7 d	–	–	–	–

Note: "– or +" stands for the gradients of dye-transmission. "–" dye-transfer remained in the primary scraped cells. "+ — +++" dye-transfer spread into 1 to 3 row of contact cells at the edge of the scraped areas, respectively.

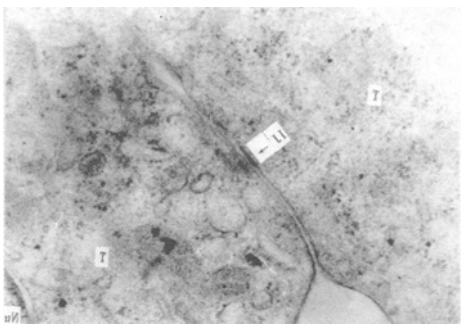

Fig. 1. The intercellular contact in CNE-2Z. IJ: intermediate junction; T: tumor cell; Nu: nucleus. EM, × 25,000

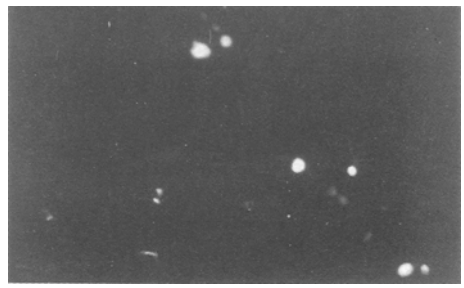

Fig. 3. LY loaded L_4 cells no dye-transfer. LM, 10×3.3

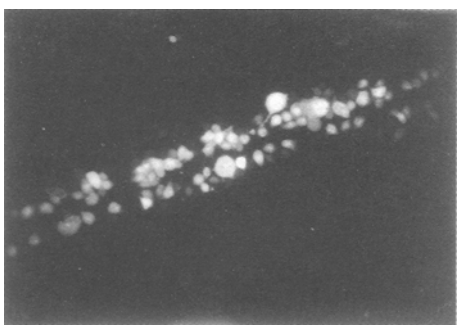

Fig. 2. LY loaded CNE-2Z cells show positive dye-transfer. LM, 10×3.3

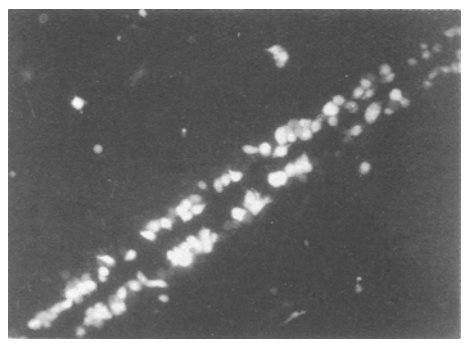

Fig. 4. CNE-2Z cells incubated in RII medium (0.001 mol/L) for 6 h show positive LY dye-transfer. LM, 10×3.3

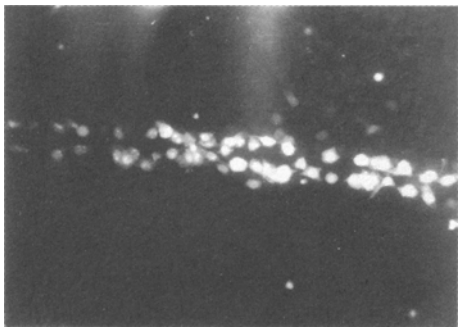

Fig. 5. CNE-2Z cells incubated in RII for 3 d show positive LY dye-transfer. LM, 10×3.3

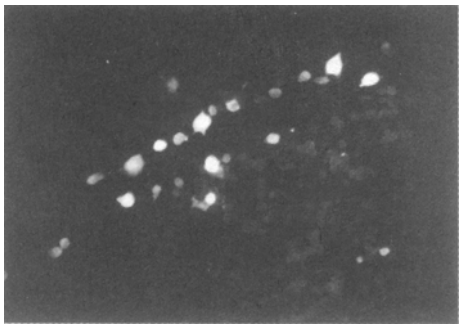

Fig. 6. CNE-2Z cells incubated in RII for 7 d. LY dye-transfer were inhibited. LM, 10×3.3

Intercellular Calcium Concentration in NPC Cells

The level of $[Ca^{2+}]_i$ in L_2 cells was significantly lower than that in CNE-2Z and H_2 cells. The concentration of $[Ca^{2+}]_i$ in L_2 cells is the highest in all kinds of all cell lines. The level of $[Ca^{2+}]_i$ in NPC cells apparently fell after 6 h of RII treatment, and rose to original level with persisting RII treatment on 7 d.

DISCUSSION

Mammalian gap junctions permit the transfer of ions and small molecules from cell to cell without leakage into extracellular space. Since gap junction mediated intercellular communication is important determinant for normal cell growth and differentiation, the loss of the ability to communicate has been suggested to be prominent in carcinogenesis by allowing potential tumor cells escape local growth control. But there are some reports on gap junctional communication in part of tumor cells. Little is known about GJIC in invasive and metastatic NPC cells. This study demonstrated that CNE-2Z cells possess GJIC with neighboring cells, while its clones do not. It may result from the biological heterogeneity of malignant cells. Although individual subpopulations or clones can result in either more or less malignant phenotypes, we could not still conclude that the difference of GJIC in NPC cell lines as a mechanism of different invasion and metastatic capacities may play a role.

There are no gap junctions observed in CNE-2Z and its variants under electron microscope. Our results seem to be contradictory with the fact that GJIC is positive in CNE-2Z. One of possible explanations is that gap junctions are not easy to be revealed in conventional thin sections, while in freeze-facture replicas gap junction may be demonstrated.[6,9]

Calcium ions are potent second messenger involved in chemical signal transductions, and also play a significant part in the regulation of gap junction permeability in tissues and cells. In this experiment, L_2 cell line showed lower concentration of cytosolic free calcium compared to H_2, L_4 cell lines. It seems to reflect that there exist some correlation between $[Ca^{2+}]_i$ level and the invasive and metastatic potentials in NPC cells lines. But the relationship between $[Ca^{2+}]_i$ level and GJIC of NPC cell was not clearly found.

4-hydroxycarbophenyl retinamide (RII) is a new synthetic analogue of retinoic acid with low toxicity. RII has been shown to inhibit the growth and invasion, and vary the expressions of oncogenes and anti-oncogenes in NPC cells.[10] We demonstrated that there was no change in the number of gap junctions in NPC cells exposed in RII. The GJIC function of CNE-2Z weakened and disappeared finally with the prolong treatment of RII. The relationship between this variation and the inhibitory effect of RII on NPC's growth and invasion has not been completely known. Most of tumor suppressor were described to stimulate GJIC, but tumor promotors inhibit GJIC between cultured cells. However our results using RII in NPC cells are not compatible with these reports. We assumed that other control mechanisms beside GJIC may be involved in tumor growth and invasion of

NPC cells. Noboru Konishi previously found that the tumor promotors (STZ, BBNa) had identical growth-promoting effects on colony formation and expansion in both NK-4 and NRK-52E cell lines, although the NK-4 line did not possess normal GJIC.[11]

Generally, gap junctional intercellular communication can be inhibited by increasing the level of intercellular calcium, thereby cell proliferation enhanced. In early duration of RII treatment, the level of intercellular calcium obviously decreased, and then rose to original level with persisting RII treatment. This founding suggested the fluctuation of $[Ca^{2+}]_i$ may be related to the effectiveness for a given period of time of RII on the growth and invasion of NPC cell lines. Whereas the mechanisms of GJIC alteration induced by intercellular calcium need to be further investigated.

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