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Development of a metastatic fluorescent Lewis Lung carcinoma mouse model: Identification of mRNAs and microRNAs involved in tumor invasion

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ABSTRACT

Cancer metastasis is the foremost cause of death in cancer patients. A series of observable pathological changes takes place during progression and metastasis of cancer, but the underlying genetic changes remain unclear. Therefore, new approaches are required, including insights from cancer mouse models. To examine the mechanisms involved in tumor metastasis, we first generated a stably transfected Lewis Lung carcinoma cell line expressing a far-red fluorescent protein, called Katushka. After in vivo growth in syngeneic mice, two fluorescent Lewis Lung cancer subpopulations were isolated from primary tumors and lung metastases. The metastasis-derived cells exhibited a significant improvement in in vitro invasive activity compared to the primary tumor-derived cells, using a quantitative invasion chamber assay. Moreover, expression levels of 84 tumor metastasis-related mRNAs, 88 cancer-related microRNAs as well as Dicer and Drosha were determined using RT-qPCR. Compared to the primary Lewis Lung carcinoma subculture, the metastasis-derived cells exhibited statistically significantly increased mRNA levels for several matrix metalloproteinases as well as hepatocyte growth factor (HGF) and spleen tyrosine kinase (SYK). A modest decrease in Drosha and Dicer mRNA levels was accompanied by significant downregulation of ten microRNAs, including miR-9 and miR-203, in the lung metastatic Lewis Lung carcinoma cell culture. Thus, a tool for cancer metastasis studies has been established and the model is well suited for the identification of novel microRNAs and mRNAs involved in malignant progression. Our results suggest that increases in metalloproteinase expression and impairment of microRNA processing are involved in the acquirement of metastatic ability.

1. Introduction

During the last decade, many new diagnostic markers for the early detection of cancer and new therapeutic drugs to treat cancer patients have been developed. Despite these advances, the dissemination of cancer cells throughout the body is hard to prevent and cancer metastasis is the leading cause of death in cancer patients (Gupta and Massague, 2006). Identification of genes and regulatory mechanisms involved in metastasizing is critical for the understanding of cancer progression and for development of new therapeutics directed against tumor metastatic capability.

Metastasis is a multistep process involving a complex interplay between tumor cells and the tumor environment. At some point, growing tumor cells from the primary neoplasm may acquire metastatic properties including loss of cell–cell adhesion, regain of mobility, and subsequent invasion into the adjacent stroma with the aid of surrounding stromal cells that provide supporting growth factors and matrix-degrading enzymes. Further dissemination of tumor cells to distant organs involves vascular intravasation. Following entry into the circulatory system and extravasation, tumor cells may start colonization of distant metastatic niches and induction of angiogenesis for sustained growth (Geiger and Peeper, 2009).

The process of tumor cell migration is in part dependent upon multiple proteolytic enzyme activities. In this regard, the family of matrix metalloproteinases (MMPs) contributes to extracellular matrix modulation, including functions such as proteolytic processing and stimulation of growth and migration. Most MMPs are zinc-dependent endopeptidases that are synthesized as inactive proenzymes and generally require proteolytic cleavage to gain catalytic activity. MMPs are expressed by a variety of cell types, including stromal and tumor cells, and are secreted or membrane-tethered proteins (Egeblad and Werb, 2002). Studies have implied an association

Abbreviations: miRNA, microRNA; HGF, hepatocyte growth factor; SYK, spleen tyrosine kinase; MMP, matrix metalloproteinase; LL-Kat, Lewis Lung carcinoma Katushka; KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, False Discovery Rate; GFP, Green Fluorescent Protein; EMT, epithelial–mesenchymal transition process; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Egfl7, EGF-like domain 7; Wwp2, WW domain containing E3 ubiquitin protein ligase 2.

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between MMP expression in cell lines and the acquisition of invasive capability (Liu et al., 2007; Zhang et al., 2006) and upregulation of MMPs has been found in various human cancers. In this regard, elevated MMP2 expression may be used as a predictor of poor cancer disease-free survival in breast and early-stage non-small cell lung cancer (Passlick et al., 2000; Talvensaari-Mattila et al., 2003).

microRNAs (miRNAs) are single-stranded non-coding RNA molecules transcribed as longer primary transcripts and cleaved in the nucleus to shorter hairpin-shaped precursor miRNAs by the RNase III endonuclease Drosha. After transport of the precursor to the cytoplasm, the mature regulatory miRNA is generated by enzymatic Dicer processing and the ≈20–23 nucleotide long miRNA is integrated into an RNA-inducing silencing protein-complex called RISC (Winter et al., 2009). Subsequently, the miRNA binds sequences in the 3′ untranslated regions of target mRNAs and suppresses mRNA translation. miRNAs participate in post-transcriptional regulation of mRNA expression in cells to maintain a balance of protein levels. Specific aberrant miRNA expression profiles for various tumors have been reported, and emerging evidence shows that deregulation of miRNA regulatory functions plays an important role in the invasion-metastatic cascade (Barbarotto et al., 2008; Zhang et al., 2010). During cancer progression, deregulation of miRNA expression may impact cellular phenotype by changing the translational efficacy of cancer-involved target mRNAs, and thereby contribute to the dissemination of cancer cells.

The purpose of the study was to derive Lewis Lung carcinoma cell subcultures after in vivo tumor growth and formation of lung metastases, in order to study the complex process of cancer progression at the molecular level in mice and thereby to identify mRNAs and miRNAs involved in tumor metastasis.

2. Materials and methods

2.1. Tumor cell line

Mouse Lewis Lung carcinoma cells (LL/2) were kindly provided by the Finsen Laboratory, Copenhagen University Hospital, Denmark. The Lewis Lung cancer cell line was grown and maintained as a mono-layer culture in RPMI1640 supplemented with 10% fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) (Invitrogen). The cells were maintained in a humidified 5% CO_2 atmosphere at 37 °C. For the experiments described, sub-confluent Lewis Lung carcinoma cells were harvested by trypsination and counted in an electronic cell counter (Nucleocounter, Chemotech). The cell line was tested free of mycoplasma.

2.2. Animals

A total of 42 female C57BL/6 mice bred in-house (Copenhagen University Hospital, Herlev) were used. Before excision of lungs or tumor cells, the mice were sacrificed by cervical dislocation. The animal experiments were conducted in accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimentation and with approval from the Danish Animal Experiments Inspectorate.

2.3. Production of a stable red fluorescent Lewis Lung carcinoma cell line

The pTurboFP635-c vector (Evrogen, Russia) was used for stable expression of the bright, far-red fluorescent protein called Katushka, which offers several advantages over GFP for visualization in live tissue (Hoffman, 2008; Shcherbo et al., 2007), under control of a CMV promoter. The plasmid was transfected into Lewis Lung carcinoma cells using the Fugene 6 system according to the manufacturer's recommendations (Roche). Stably transfected Lewis Lung carcinoma Katushka cell clones were obtained by dilution of the cells and selection with 800 μg/ml Geneticin (G418) (Sigma-Aldrich). The expression of Katushka was verified by epifluorescence microscopy (Diaplan Microscope, Leitz) using a TRITC filter. Pictures were taken with a Nikon DXM 1200C camera.

2.4. Establishment of a primary tumor-derived and metastasis-derived Lewis Lung carcinoma Katushka cell culture

Four C57BL/6 female mice were inoculated subcutaneously with 0.5×10^6 Lewis Lung carcinoma Katushka cells in 100 μl PBS in the flank to establish the tumors. After 15 days of tumor development, the mice were sacrificed and primary tumors growing in the flank of the mice were sterilely harvested and pooled from the four mice. Tumor tissue was minced with a scalpel on ice and further dissociated through a needle. The lungs from the mice were sterilely isolated and atypical appearances (tumor metastases) on the lung surfaces were isolated, pooled from the four mice and dissociated in PBS on ice using a needle. Primary tumor and metastatic tumor material was plated for in vitro culture. After 12 days, selection for Lewis Lung carcinoma Katushka (LL-Kat) cells was started by addition of G418 to the two growing cell cultures. Subsequently, after selection, cells were expanded before further analysis.

2.5. Analysis of Lewis Lung carcinoma metastases

For examination of the lung metastatic capability of LL-Kat cells, mice were injected with LL-Kat cells in the flank and after tumor growth, the mice lungs were excised and fixed in Bouin's solution (Sigma Aldrich). Tumor nodules were examined under a dissecting microscope.

2.6. MTT assay

Lewis Lung carcinoma cells were seeded onto 96-well microculture plates at a density of $2–4\times10^3$ cells/well in 100 μl growth medium and incubated at 37 °C for 1–4 days. After incubation, 50 μg MTT (Hospital Pharmacy, Herlev, Denmark) was added to each well and cells were further incubated at 37 °C. After 4 h the reaction was terminated by adding 100 μl 10% SDS in 0.01 M HCl (Hospital Pharmacy, Herlev) and purple formazan crystals were solubilized overnight at 37 °C. The absorbance was measured at 550 nm with a background subtraction at 690 nm in a Multiskan Ascent absorbance plate reader (Thermo Labsystems).

2.7. Determination of invasive capability of Lewis Lung carcinoma cells

The invasive capability of Lewis Lung carcinoma cells was measured using the BioCoat Tumor invasion system (BD Biosciences) according to the manufacturer's instructions. 24-well insert plate with an 8 μm pore FluoroBlok membrane blocking the passage of light from 490 to 700 nm coated with basement membrane proteins was used. In brief, Lewis Lung carcinoma cells were labeled in situ for 1 h at 37 °C with 10 μg/ml of DilC12 (BD Biosciences) in medium containing 10% serum. After labeling, the cells were trypsinized, counted and a total of 5×10^4 cells in serum-free medium were seeded in each insert well. Medium containing 10% serum was used as chemoattractant. Cells were incubated 17–42 h at 37 °C in a humidified incubator to allow cells to migrate and invade the matrigel membrane. Successfully penetrating cells were quantified by a bottom reading fluorescence plate reader at excitation/emission wavelengths of 530/590 nm with a background subtraction measured at 0 h (SynergyTM HT Multi-detection microplate reader, BioTek instruments). Appropriate fluorescence intensities of plated cells were ensured using a top reading measurement at 0 h.