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Increased μ-opioid receptor expression in metastatic lung cancer

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Editor's key points

- In laboratory cell lines and animal models, μ-opioid receptor (MOR) overexpression promotes tumour growth and metastasis.
- This study evaluated the association between MOR expression and metastasis in archived samples of lung cancer.
- MOR overexpression was evident in lung cancer samples of patients with metastasis compared with lung cancer patients who did not have metastasis.

Background. We and others have previously demonstrated that the μ-opioid receptor (MOR) is overexpressed in several human malignancies. There is a seven-fold increase in MOR in cell lines of human lung cancer. In animal models, overexpression of MOR promotes tumour growth and metastasis. We, therefore, examined whether MOR expression is increased in metastatic lung cancer.

Methods. In this study, we examined the association between MOR expression and metastasis in archived biopsy samples from patients with lung cancer. Paraffin-embedded patient material was stained using MOR antibody and scored qualitatively by two independent pathologists using a four-point scale.

Results. In human lung cancer and normal adjacent lung samples obtained from 34 lung cancer patients, MOR expression was increased significantly in cancer samples from patients with lung cancer compared with adjacent control tissue ($P=0.0242$). When the samples from patients with metastatic lung cancer were separated from the cohort of the total number of patients with lung cancer, we observed an approximately two-fold increase in MOR expression ($P=0.0013$).

Conclusions. The association between the expression of MOR and the progression of the tumour is consistent with the hypothesis of a direct effect of MOR on cancer progression.

Keywords: lung cancer; metastasis; methylnaltrexone; MOR; μ-opioid receptor

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The role of anaesthesia and analgesia in the recurrence and metastatic rate of malignancies has recently received considerable attention.[1–4] Retrospective studies have demonstrated a lower incidence of cancer recurrence after regional anaesthesia with low doses of opioids after surgery for breast, prostate, colon cancer, and melanoma; other studies have failed to detect significant differences.[5–8] Explanations for the differences in recurrence rates include immune suppression and direct effects on tumour cell growth.[9–11] Our research has focused on the μ-opioid receptor (MOR) in directly regulating tumour growth and metastasis.[12 13]

Effective therapeutic strategies for lung cancer, the leading cause of cancer-associated mortality worldwide, are extremely limited exemplifying the need for early diagnosis and novel therapeutic interventions.[14 15] We have previously reported that the MOR is upregulated in several types of human non-small-cell lung cancer (NSCLC).[13] In xenograft models, overexpression of the MOR in human NSCLC increased primary tumour growth and metastasis.[12] The correlation between the MOR and metastatic lung cancer, however, has not been previously reported in patient samples.

Several targeted therapies in NSCLC exist including epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (gefitinib and erlotinib) and monoclonal antibodies (cetuximab).[16–19] The overall survival rate for NSCLC, however, still remains low.[20–22] Fujioka and colleagues found that morphine can stimulate EGFR-signalling pathways such as the serine/threonine kinases Akt and MAP kinase in NSCLC. This result suggests that MOR inhibition is a potential therapeutic strategy for NSCLC.[23] In another recent study, MOR expression correlated with progression-free and overall survival in prostate cancer.[24]

Based on the recent interest in the effects of anaesthesia and analgesia regimens on the recurrence and metastatic potential of various cancers,[1–4] published data that the MOR is upregulated in lung tissue from patients with NSCLC,[13] that overexpression of MOR promotes tumour growth and metastasis in human NSCLC xenograft models,[12] and that MOR expression correlates with prostate cancer survival,[24] we investigated MOR expression in lung cancer patient samples.

Methods

Lung cancer tissue samples

We used tissue samples from human subjects that were cytologically or histologically documented with NSCLC. The tissues were archival surgical specimens already banked in the University of Chicago Lung Cancer Tissue Bank or in the pathology department. The tissues were obtained with the subjects' written informed consent under protocols 9571 and 13 473. The protocols give access to clinical information for the tissues in this study. The University of Chicago IRB grants a waiver of consent for archival tissues from patients who have died (10654N), and the protocol also gives access to the associated clinical data from the tissue bank database. Tumour tissues not associated with personal information (de-identified) that have already been banked were used under protocol 10653N. Use of these previously stored samples was granted exemption status through the University of Chicago IRB under PHS exemption category number 4. All data, which included patient, clinical, and treatment outcomes, were entered into our HIPAA regulated database.

MOR immunohistochemistry

Samples for this study were acquired from paraffin-embedded, formalin-fixed tissue archived at The University of Chicago (Chicago, IL, USA) Human Tissue Resource Center. Immunohistochemistry for human MOR was conducted on tissue sections using rabbit anti-MOR antibody (GeneTex, San Antonio, TX, USA) that was previously validated in our laboratory.[12 13 25–28] Following an antigen retrieval protocol, tissues were deparaffinized, blocked with BSA, probed with primary and secondary antibody. Tumour sections were evaluated by a pathologist who was blinded to the identity of the tissue as previously described.[25 26] Briefly, IHC score was obtained by the semi-quantitative method that accounts for staining intensity and percentage of cell stained. This estimation resulted in an IHC score of 0, 1, 2, or 3 denoting negative, weak, moderate, and strong expression, respectively. This score was correlated with available clinical information in future analyses. Images were captured at $\times 20$ or $\times 40$ using a Fisher Scientific Micromaster digital microscope (Pittsburg, PA, USA) and the Micron USB live-field capture software (Westover Scientific, Mill Creek, WA, USA) as previously described.[25 26]

Statistical analysis

For individual groups, normal distribution probabilities and *P* values were calculated using Lilliefors test for normality.[27] For differences in MOR staining intensity in patient samples between normal adjacent control, total lung cancer, and subset of lung cancer with lymph node metastasis, data were compared by unpaired Student's *t*-test. $P<0.05$ was considered statistically significant. Statistical analysis was performed using the GraphPad Prism program (GraphPad Software Inc., La Jolla, CA, USA). Other graphical quantitation was performed using the Microsoft Excel™ software.

Results

MOR staining intensity was increased in lung cancer patient samples *vs* normal adjacent controls (1.24 *vs* 0.74, $P=0.0242$) (Table 1). The sets of MOR staining for normal adjacent control

Table 1 Comparison of various parameters between normal adjacent control, total lung cancer, and subset of lung cancer with lymph node metastasis patient samples. MOR immunohistochemistry was performed on de-identified lung cancer patient samples and scored by two independent pathologists on a four-point scale (0, 1, 2, 3) as described in the Methods. Various parameters were analysed including MOR staining intensity, age, gender, NSCLC subtype histology, and MOR staining by histology. Adeno, adenocarcinoma; Large cell, large-cell carcinoma; SCC, squamous cell carcinoma. For certain parameters, data means, data ranges, and data *P* values are presented. *P*-value within groups: **P* values of individual datasets using the Lilliefors test for normality.[27] *P*-value between groups: ***a* comparison between either normal adjacent control and lung cancer or lung cancer and subset of lung cancer patients with lymph node metastasis using Student's *t*-test

	Normal adjacent control ($n=34$)		**Lung cancer ($n=34$)**			**Subset of lung cancer patients with lymph node metastasis ($n=7$)**		
Parameter	**Mean (range)**	***P*-value within groups***	**Mean (range)**	***P*-value within groups***	***P*-value between groups****	**Mean (range)**	***P*-value within groups***	***P*-value between groups****
MOR staining intensity	0.74 (0–2)	0.001	1.24 (0–3)	0.001	0.0242	2.0 (1–3)	0.008	0.0013
Age		67.8 (46–90)		0.186	N/A	70.4 (61–81)	0.5	0.236
Gender			Male=17 Female=17				Male=5 Female=2	
Stage			0–3				2–3	
Histology			Adeno=14 Large cell=5 SCC=15				Adeno=4 SCC=3	
MOR staining/ histology	Adeno=0.87 (0–2) Large cell=0.80 (0–2) SCC=1.00 (0–2)	0.022 0.5 0.027	Adeno=1.00 (0–3) Large cell=1.60 (0–3) SCC=1.07 (0–2)	0.001 0.470 0.01	0.732 0.242 0.857	Adeno=2.0 (1–3) SCC=2.0 (2–2)	0.5 N/A	N/A N/A

and lung cancer each had a normal distribution as determined by the Lilliefors test for normality[27] (P=0.001 and P=0.001). When broken down into NSCLC subtypes, the largest differences in MOR staining, while statistically not significant, were observed in large cell carcinoma (1.60 *vs* 0.80, P=0.242), then in adenocarcinoma (1.00 *vs* 0.87, P=0.732), and finally in squamous cell carcinoma (1.07 *vs* 1.00, P=0.857).

We next examined the samples from a subset of total lung cancer patients with lymph node metastasis. The MOR staining for this subset of total patients with lung cancer had a normal distribution as determined by the Lilliefors test for normality[27] (P=0.008). Samples from lung cancer patients with lymph node metastasis had higher MOR staining intensity than did the samples in the total lung cancer cohort (2.00 *vs* 1.24, P=0.0013). The average patient age was similar in the two groups (70.4 *vs* 67.8 yr, P=0.236). The metastatic portion was derived from adenocarcinoma and squamous cell carcinoma subtypes of NSCLC.

We examined MOR IHC stained sections from NSCLC patients with Stage 0, 1, 2 and 3 adenocarcinoma (Fig. 1A–D). MOR staining intensity increased with cancer stage. The pattern of MOR staining appeared to change from a nuclear (Fig. 1A) to cytosolic (Fig. 1B) to a cytosolic/membrane cellular localization (Fig. 1C and D). This is consistent with prior observations in prostate cancer.[24] We also examined lymph nodes from NSCLC adenocarcinoma patients with or without cancer cell invasion. MOR

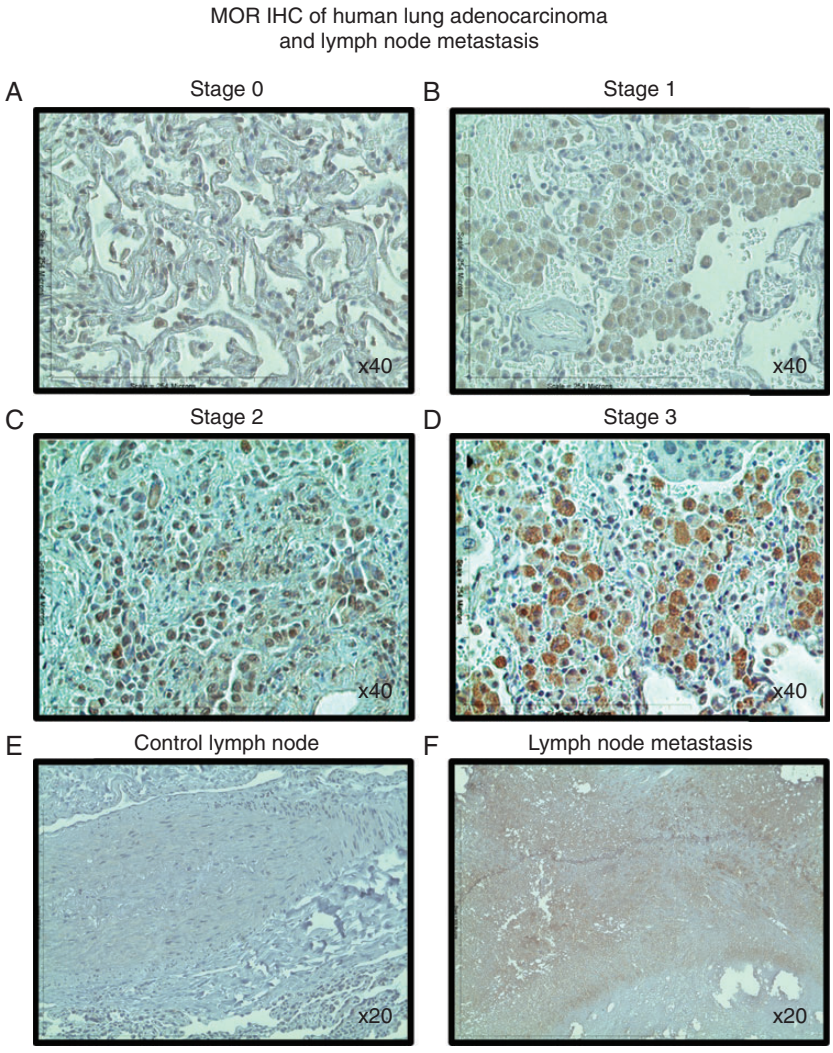

Fig 1 MOR immunohistochemical staining intensity increases with NSCLC tumour stage and lymph node metastasis in patient samples. Patient NSCLC adenocarcinoma lung cancer samples were acquired from paraffin-embedded, formalin-fixed tissue archived at The University of Chicago (Chicago, IL, USA) Human Tissue Resource Center. Immunohistochemistry for human MOR was conducted on tissue sections and images were captured at ×20 or ×40 using a Fisher Scientific Micromaster digital microscope and the Micron USB live-field capture software. MOR staining intensity increases with cancer stage (A–D). The pattern of MOR staining appears to change from a nuclear (A) to cytosolic (B) to a cytosolic/membrane cellular localization (C and D). MOR staining intensity is also increased in metastatic lymph nodes (E *vs* F).

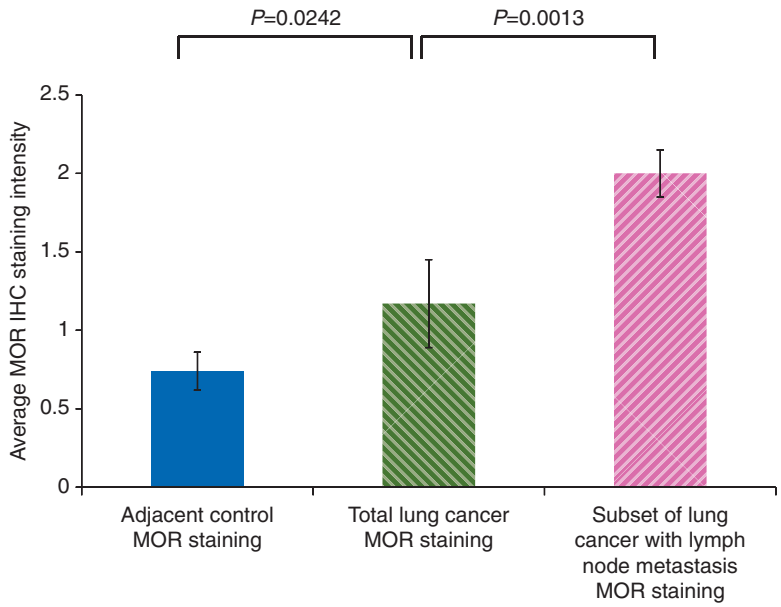

Fig 2 Graphical representation of the average MOR immunohistochemical staining intensity of normal adjacent, total lung cancer, and subset of lung cancer with lymph node metastasis patient samples. MOR immunohistochemistry was performed on de-identified normal adjacent control and lung cancer patient samples, scored by two independent pathologists on a four-point scale (0, 1, 2, 3) and box plots were generated as described in the Methods. There was a statistically significant difference between normal adjacent control and total lung cancer (P=0.0242) and also total lung cancer and subset of lung cancer with lymph node metastasis (P=0.0013).

staining intensity was also increased in metastatic lymph nodes (Fig. 1E *vs* F).

Although the difference between normal adjacent control and total lung cancer MOR staining was statistically significant, there was considerable overlap (0.74 *vs* 1.24, P=0.0242) (Table 1). We, therefore, performed a statistical analysis of the average MOR staining intensity of the patient samples between total lung cancer and the subset of total lung cancer with lymph node metastasis. Analysis in this fashion yielded a much larger significant difference in staining intensity between total lung cancer and the subset of total lung cancer with lymph node metastasis (1.24 *vs* 2.0, P=0.0013) (Fig. 2). Although our sample size was relatively small, our results are consistent with the hypothesis of a direct effect of MOR on cancer progression.

Discussion

Our most important finding that samples from patients with metastatic disease exhibited a dramatically higher MOR expression serves as a bridge between prior molecular studies and recent clinical observations in humans. Our observations specifically support other molecular, cellular, and animal evidence demonstrating that the μ-opiate receptor plays an important role in the progression of lung cancer.

In preclinical models, μ-opioids stimulate angiogenesis and tumour progression through the MOR.[11 13 29 30] Gupta and colleagues initially reported that μ-opioids at clinically relevant doses were proangiogenic in a model of breast cancer xenografts.[28] Support for the hypothesis that the MOR is involved in cancer progression comes from other work showing a reciprocal transactivation of the VEGF receptor,[31] and potentiation of bevacizumab and 5-fluorouracil[30] and also mTOR inhibitors[29] in human endothelial cells by the peripheral opiate antagonist methylnaltrexone (MNTX). In MOR knockout mice, there was markedly diminished progression of Lewis lung carcinoma, and MNTX or naltrexone infusions blocked tumour growth and metastasis.[13] Tumour progression appeared not to be mediated through the effects of opioids on the immune system as it was not significantly influenced in nude mice.[12 13] Although several studies have failed to implicate an association between the MOR and progression, in at least one of the studies, pharmacologic rather than clinical doses of opioids were used.[30] MNTX blocked several intermediaries of cancer progression including Src even absent exogenous opiates,[32] suggesting a dual effect on the angiogenic pathway.

In addition to the *laboratory* evidence supporting involvement of the MOR in cancer progression, results of some *human* studies are consistent with this finding. Recently, in a retrospective study of 113 patients with advanced prostate cancer, μ-opiate receptor expression and opioid requirement were independently associated with reduced progression-free survival and overall survival.[24] These observations in prostate cancer patients are remarkably consistent with what we have demonstrated in our cohort of lung cancer patients and suggest a broader applicability of the hypothesis that the MOR is involved in cancer progression. In our study, the

increase in the MOR was especially prominent in samples with metastatic disease.

Further human evidence supporting the role of the MOR in cancer progression in humans comes from a recent study examining the A118G gene mutation of the MOR.[33] In that retrospective study of 2039 women with breast cancer, the A118G gene polymorphism, which decreases response to opioid receptors, was associated with a two-fold improvement in survival in heterozygotes and a four-fold difference in survival in homozygotes at all stages of disease. The role of the A118G MOR gene mutation in tumour progression was confirmed in oesophageal cancer.[34]

Finally, support for an effect of opioids on human cancer comes from a study[35] of palliative care patients: intrathecal fentanyl markedly improved survival compared with medical treatment with systemic opiates. In a study of three patients with pancreatic cancer treated with α-lipoic acid and low-dose naltrexone, the proliferation of malignant cells was attenuated.[36]

Although there is growing evidence for an effect of the MOR in mediating tumour metastasis, the reason for this effect remains unclear. Our studies of tumour progression were performed in the absence of exogenous opiates, suggesting an intrinsic process. Studies in human melanoma showed that endogenous opioid expression is associated with tumour progression.[37] μ-Opioids reduced cell–cell adhesion in a concentration-dependent manner in layers of human pulmonary endothelial cells and facilitated capillary leakage in animal models.[38] Opioids at clinically relevant doses potentiated epithelial mesenchymal transition, and opioid receptor antagonists blocked both opioid- and EGF-mediated changes in that process.[39] Thus, it is possible that the endothelial barrier dysfunction in cellular and animal models may permit further seeding of the existing tumours.

Although our sample sizes were small, the individual sets of MOR staining samples for normal adjacent control, lung cancer, and subset of lung cancer with lymph node metastasis each had a normal distribution as determined by the Lilliefors test for normality.[27] Further, there was a statistically significant difference using Student's *t*-test between the groups. However, a larger sample size will be needed to conduct advanced statistical analyses.

The present study has focused on cancer patients, but our findings could provide an explanation for the observations that cancer recurrence is associated with the type of anaesthesia used in surgery. There are important differences between opioid use for surgery in opiate naive individuals and chronic use of opioids in cancer patients. The physiologic observations we have made are consistent with the initial findings in breast and lung cancer patients.[5 6] The only prospective study in this area failed to validate that the type of anaesthesia had a radically different effect in colon cancer patients,[8] and the proof of a direct effect of anaesthetic techniques in cancer surgery awaits results of prospective trials. In conclusion, our study extends our observations that opioids and the opioid receptor affect tumour progression in humans and suggests a potential use of MOR antagonists as a therapeutic option in tumour treatment.

Authors' contributions

P.A.S., R.S., and J.M. conceived of the strategies and supervised the project. P.A.S. and T.M. designed and performed experiments and analysed data. P.A.S. and J.M. wrote the manuscript. R.H. designed and performed experiments.

Declaration of interest

MNTX was developed at the University of Chicago and licensed to Progenics Pharmaceuticals, subsequently sub-licensed to Salix Pharmaceuticals. J.M. was a paid consultant for Progenics Pharmaceuticals and currently is a paid consultant for Salix Pharmaceuticals. He receives royalties through the University of Chicago.

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