Reply to Reviewers

Reviewer

This an interesting case report, in which the authors document EGFR mutation in an osteolytic lesion in the femur, and trace its origin back to an adenocarcinoma of the lung. The manuscript is quite well written and presented. I have a few suggestions to improve the clarity and scope of the paper.

1. The sequence data shown in Fig 4A need to be improved – the read is "N" i.e. unidentifiable, compared to "G" in Fig. 4B. As the crux of the manuscript is that the same mutation was present in primary and metastatic lesions, this must be unequivocal.

Reply: The software reads "N" when two nucleotides are present equally. As showing by the arrows, "G" and "A" were observed in both tumors (Figs 4A and B), which means point mutation from "GGC" to "AGC" on codon 719 in both tumors. To make it understand easily, I deleted displaying the nucleotide sequences read by the software, and added "GGC→AGC" on Fig. 4.

2. Introduction: spell out / explain the tumor markers (CEA, CYFRA, SCC).

Reply: We spelt them out according to the suggestion.

3. Fig. 2C&D – show appropriate negative controls. Which isoform of p63 was detected?

Reply: Because adenocarcinoma samples from the lung must be the best negative controls in this case, I added Fig. 3C and D as the negative controls for Fig. 2C and D, respectively. Anti-p63 antibody used is from clone 4A4 (Nichirei Biosciences Inc., Tokyo, Japan). This antibody detects whole p63, but not any specific isoform of p63.

4. A brief description of the antibody staining methods (see point 2, above) is required.

Reply: All immunostains were performed according to the manufacturer's recommendation. Brief information about antibodies was added in the Figure 2 legend.

5. The paper would benefit from some additional discussion regarding squamous vs. adenoid differentiation and the factors involved. Also, are there any previous studies of

similar nature that have been performed on primary/metastatic lesions in lung or other tissues, e.g. using p53 mutations? This would be worth including.

Reply: We thank for these useful comments. Regarding the mechanisms of histological differentiation, some interesting studies have been reported. Lung adenocarcinoma phenotypes have been reproduced by introducing an active form of PIK3CA, CYCLIN-D1, or a dominant-negative form of LKB1 in combination with genetic alterations including hTERT overexpression, inactivation of the pRB and p53 pathways, and KRAS activation [Sasai K, 2011]. p63 regulates cell proliferation and differentiation [Truong AB, 2006] and is a well-known marker of squamous differentiation [Conde E, 2010]. Although the actual factors involved in squamous and adenomatous differentiation in the current case are unknown, these molecular mechanisms may have been involved. We added this discussion in the manuscript (DISCUSSION, 3rd paragraph).
In addition, we found several previous reports showing the usefulness of p53 analysis in primary and metastatic lesions. In one study, a p53 mutation analysis determined that the ovarian tumor was a metastasis from the sigmoid colon [Yamano T, 2010]. Similarly, an identical p53 mutation confirmed that a neck nodal metastasis originated from oral squamous cell carcinoma that had been treated over five years prior [Hoekstra JW, 2008]. We included these previous reports in the manuscript and added some discussion on usefulness of genetic analysis (DISCUSSION, 5th paragraph). Genetic analyses could help determine the origin of tumors of so-called unknown origin.

We added next five references.

Sasai K, Sukezane T, Yanagita E, Nakagawa H, Hotta A, Itoh T, Akagi T. Oncogene-mediated human lung epithelial cell transformation produces adenocarcinoma phenotypes in vivo. *Cancer Res* 2011; **71**: 2541-2549 [PMID: 21447735 DOI: 10.1158/0008-5472.CAN-10-2221]

Truong AB, Kretz M, Ridky TW, Kimmel R, Khavari PA. p63 regulates proliferation and differentiation of developmentally mature keratinocytes. *Genes Dev* 2006; **20**: 3185-3197 [PMID 17114587]

Conde E, Angulo B, Redondo P, Toldos O, García-García E, Suárez-Gauthier A, Rubio-Viqueira B, Marrón C, García-Luján R, Sánchez-Céspedes M, López-Encuentra A, Paz-Ares L, López-Ríos F. The use of P63 immunohistochemistry for the identification of

squamous cell carcinoma of the lung. *PLoS One* 2010; **5**:e12209 [PMID: 20808915 DOI: 10.1371/journal.pone.0012209]

Yamano T, Morii E, Arai I, Takada T, Kubota K, Sato M, Inoue T, Okada Y, Hara T, Aozasa K. Diagnosis of primary versus metastatic ovarian adenocarcinoma using p53 gene mutation analysis. *Int J Clin Oncol* 2010; **15**: 621-625 [PMID: 20514505 DOI: 10.1007/s10147-010-0096-z]

Hoekstra JW, Kummer JA, van Es RJ. Late (> 5 years) regional lymph node metastasis of oral squamous cell carcinoma (SCC), proven by p53 mutation analysis. *J Craniomaxillofac Surg* 2008; **36**: 415-418 [PMID: 18554920 DOI: 10.1016/j.jcms.2008.04.002]

Reviewer
The authors reported a case that a 70-year-old Japanese woman had an adonocarcinoma in her lung and squamous carcinoma in her femur. They identified EGFR mutation, G719S, in both tumors, and then they conclude the tumor in the femur was identified genetically as a lung cancer metastasis, and both tumors had a common origin, despite their histologic dissimilarity. However if the authors have the evidence to show that in general there is no EGFR mutation, G719S, in the tumor in the femur, then the inference can be drawn.

Reply: The reviewer's question is fundamental and important. If normal tissues or tumors in the bone often have mutations in *EGFR*, the present case is not worthy to be reported. In general, *EGFR* mutations have been reported to be almost exclusively found in carcinomas of the lung, although they are observed with a low frequency in other solid tumors [Masago, 2009]. There is no report showing *EGFR* mutation in the tumor in bones except for metastasis from the lung cancer. G719S is an uncommon *EGFR* mutation. No G719S mutation has been reported in solid tumors other than lung cancer. It is important that primary lung cancer did exist in the present case. We added this discussion in the manuscript with a reference below (DISCUSSION, the first paragraph).

Masago K, Asato R, Fujita S, Hirano S, Tamura Y, Kanda T, Mio T, Katakami N, Mishima M, Ito J. Epidermal growth factor receptor gene mutations in papillary thyroid carcinoma. *Int J Cancer* 2009; **124**: 2744-2749 [PMID: 19253367 DOI: 10.1002/ijc.24250]