diagnosis and identified vascular and perineural invasion with metastasis to the peritumoral lymph nodes. The patient started chemotherapy at that time. Our main question was whether the new lesions were metastases of the rectal tumor or corresponded to a new tumor (e.g., bone sarcoma with pulmonary and hepatic metastases), which would imply the need to change the treatment strategy. Biopsies of the bone lesion and a pulmonary nodule were performed, and showed metastatic adenocarcinoma with a tubuloacinar pattern (Fig. 1D).

Calcification of a pulmonary nodule is usually suggestive of its benign nature – such nodules are most commonly granulomas and less commonly hamartomas – but calcification and ossification can also occur in malignant lesions. Multiple pulmonary nodules have numerous etiologies, but the diagnostic possibilities are considerably reduced when these lesions show calcification. The main diagnostic considerations are calcified pulmonary metastases, amyloidosis, hyalinizing granulomas, epithelioid hemangioendothelioma, necrobiotic nodules, and multiple chondromas. Amyloidosis, in its nodular form, is usually asymptomatic. The definitive diagnosis is made by histopathology, on the basis of the finding of deposition of amyloid, which stains with Congo red and shows apple-green birefringence under polarized light. Hyalinizing granulomas are rare fibrotic pulmonary lesions, usually associated with autoimmune phenomena related mainly to exposure to mycobacterial or fungal antigens. Epithelioid hemangioendothelioma is a rare multifocal pulmonary neoplasm of endothelial origin. It is considered to be a sarcoma of low aggressiveness. Necrobiotic nodules can develop in patients with pneumoconiosis associated with rheumatoid arthritis. Calcification in pulmonary chondromas is a common radiological finding. The association of these chondromas with gastrointestinal stromal tumors and extra-adrenal paragangliomas is known as the Carney triad.[1–3]

The calcification of pulmonary metastases is very uncommon. The tumors that most commonly give rise to calcified metastases are sarcomas (osteosarcoma, chondrosarcoma, synovial sarcoma, and giant cell tumor of the bone), carcinomas (particularly mucinous and papillary adenocarcinomas), and treated metastatic choriocarcinoma. Several mechanisms are responsible for the calcification of metastases: bone formation in tumor osteoid in osteosarcoma; calcification and ossification of tumor cartilage in chondrosarcoma; dystrophic calcification in papillary carcinoma of the thyroid, giant cell tumor of the bone, synovial sarcoma, and treated metastatic tumors; and mucoid calcification in mucinous adenocarcinoma of the gastrointestinal tract and breast. Calcification can develop in metastases of several other tumors after chemotherapy or radiotherapy, generally secondary to degeneration, hemorrhage, and necrosis.[1,4,5] Although tubular-type adenocarcinoma is not listed among the major causes of calcified metastases, the patient described here had undergone previous chemotherapy, which may have been the mechanism for calcification formation.

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Changes in the Melting Peak of Hybridization Probes Used for Genotyping in Alpha-1 Antitrypsin Deficiency Do Not Always Imply Errors☆

Las alteraciones en el pico de fusión de las sondas de hibridación usadas para el genotipado en la deficiencia de alfa-1 antitripsina no siempre implican errores

To the Editor,

Molecular analysis of the gene that encodes alpha-1 antitrypsin (AAT; *SERPINA1* gene) is the gold standard for the identification of allelic variants.[1] The different molecular methods that can be used for this purpose include hybridization probes or HybProbes,[2] which provide real-time PCR tracking. Once the amplification process is complete, these probes identify the genetic variants present in a particular region within the amplicon. This is a homogeneous genotyping test, that is to say, the entire process occurs in a single tube with no additional manipulation between the start of the test and the observation of the results. However, while it is a very reliable technique, errors may sometimes occur, especially in the interpretation of the results.[3]

We performed an analysis of the prevalence of non-S/S and non-Z/Z variants of the *SERPINA1* gene in a clinical population from La Palma (Canary Islands, Spain) by recruiting a series of 1510 patients regardless of the reason that led them to the pulmonology clinic. We identified 7 subjects in whom the peaks in the melting charts displayed by the HybProbe probes designed to identify the non-S/S variants showed a shift in respect of normal charts (Fig. 1). These 7 patients had been diagnosed with various respiratory diseases, such as diffuse interstitial lung disease, sleep apnea–hypopnea syndrome, and chronic obstructive pulmonary disease.

To rule out an error in the genotyping process due to differences in the saline concentration of the 7 DNA samples involved, these were prepared and analyzed again. In the new analysis, the real-time PCR genotyping platform software (LightCycler 480) continued to allocate these samples to a different genotype group than those defined by the standards, using the computer application's

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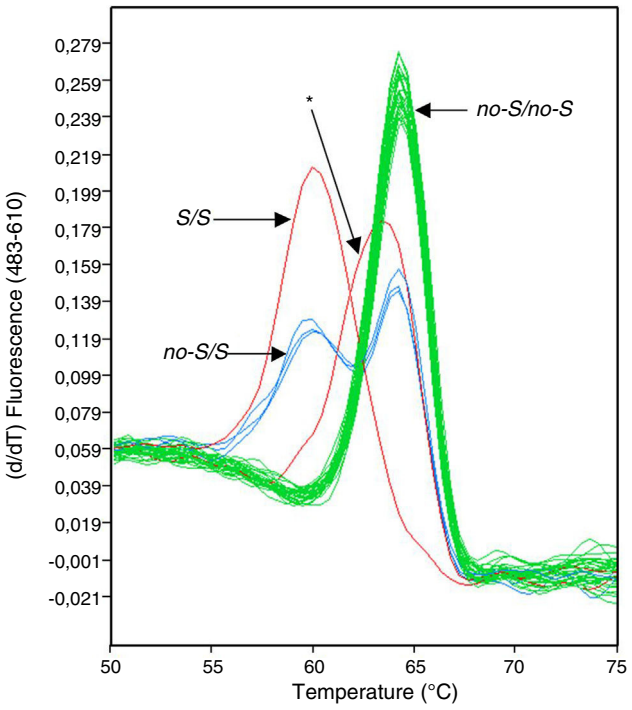

Fig. 1. Melting analysis with HybProbe probes designed to detect non-S and S variants in the *SERPINA1* gene. Genotype groups recognized by the test software are indicated by arrows. The asterisk indicates the anomalous melting peak.

default threshold values for similarity and resolution. A review of the melting temperatures indicated a clear difference between the melting peaks of the 7 samples (average=63.21 °C; SD=0.05) and the closest melting peaks from samples with a non-S/S genotype (mean=64.18 °C; SD=0.26; *n*=71).

These results suggested that the *SERPINA1* gene in those individuals contained a mutation other than the S variant (c.863A>T; p.Glu264Val) in the region covered by the genotyping probes. One candidate was the c.839A>T (p.Asp256Val) mutation, a severe deficiency variant with clinical implications that defines, depending on the genetic background, the alleles PI^*P_{lowell}, PI^*P_{duarte} and $PI^*Y_{barcelona}$.[4–6] This possibility was ruled out when it was found that serum AAT levels of 7 patients were in the range of 93.5–167 mg/dl. Despite these patients had not yet shown signs of severe AAT deficiency, we decided to continue investigating what appeared to be the most frequent rare genetic variant in our clinical sample. In this respect, the samples of 3 of the 7 patients underwent amplification of the coding region of the *SERPINA1* gene and the corresponding introns, in the form of two overlapping amplicons, which were sequenced. This analysis revealed that the 3 patients were carriers of the c.840T>C (p.Asp256Asp) mutation in heterozygosis, specifically in the region to which the anchor probe used to detect the non-S/S variants was attached. Moreover, one of these patients was heterozygous for the c.774G>A (p.Lys236Lys) mutation. These mutant variants appear not to be deficiency variants, since the levels of AAT measured in PI^*MM_{D566D} patients (166 and 142 mg/dl) and in the PI^*SM_{D566D} patient (125 mg/dl) fit perfectly within the reference range for individuals with genotype *PI*MM* or *PI*MS*.[7] This observation is predictable, since these are 2 mutations that do not change the protein sequence (synonymous mutations), and can also be expected not to affect the analysis of the phenotype of these patients by isoelectric focusing.

The c.840T>C mutation, in the normal M1-Val213 allelic background, in combination with the c.1093G>A (p.Asp341Asn) mutation make up the non-deficiency allele $PI^*P_{saint\ albans}$.[4] Since this second mutation was not found in the 3 patients analyzed, we ruled out the presence of the allele in these individuals. Since conventional PCR simultaneously amplified the 2 alleles of the *SERPINA1* gene, we could not determine exactly in which normal allelic background the mutations detected in these patients were located. However, we could deduce which combination of these allelic backgrounds were present in each patient. Interestingly, the c.840T>C mutation in the M1-Ala213 background, or the combination of c.840T>C and c.774G>A mutations in the M1-Val213 background, have only been described to date in the sub-Saharan population from South Africa, where they reach allele frequencies of 10.4% and 4.3%, respectively.[8] Although confirmation would require haplotype analysis, these sub-Saharan alleles might be found in the current population of the island of La Palma and, presumably, were incorporated via African slaves brought to the island after the mid-16th century.[9]

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