Identification of levers for improving dead equine traceability: A survey of French equine owners' perception of regulatory procedures following their animal's death

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Hepatitis B virus replicating in hepatocellular carcinoma encodes HBx variants with preserved ability to antagonize restriction by Smc5/6

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List of Abbreviations:

HBV: Hepatitis B Virus; pgRNA: pregenomic RNA; RC-DNA: relaxed-circular HBV DNA cccDNA: covalently closed circular HBV DNA; Smc5/6: structural maintenance of the chromosome proteins 5/6; vp: viral particles; MOI: multiplicity of infection; dHepaRG: differentiated HepaRG; CCNA2: cyclin A2; ORFs: open reading frames; T: tumor; NT: non tumor ; FL-HBx: full length HBx; HTLV-1: T-cell lymphotropic virus type 1; ALT :T-cell leukemia/lymphoma .

Abstract

Hepatitis B virus infection is a major cause of liver diseases including hepatocellular carcinoma (HCC). The viral regulatory protein HBx is essential for viral replication and has been involved in the development of HCC. Recently, we characterized a subset of HCCs that replicate HBV. Our aim was to characterize HBx encoded by the full-length HBV DNA (cccDNA) in HCC and non-HCC liver. HBx genes were amplified and sequenced from eight paired HCC and non-HCC tissues in which HBV cccDNA and pgRNA were both present. Sequence analyses identified twelve amino acid positions mutated between HCC and non-HCC liver, and detected in at least three cases. We next assessed the impact of these mutations on HBx function by testing their transcriptional activity. We examined their ability to rescue the transcription of HBV virus deficient for HBx in differentiated HepaRG cells and to induce Smc5/6 degradation, which is mandatory for viral replication. We assessed their capacity to activate a CREB-dependent reporter. Finally we analyzed their growth suppressive activity using colony formation assays. Our results showed that most HBx variants isolated from HCC retain their ability to support HBV cccDNA transcription and to degrade Smc5/6. Strikingly, HCC specific HBx variants are impaired in their antiproliferative activity, which may be detrimental for tumor growth. In conclusion, in contrast to previous observations that tumor HBx variants lack transcriptional activity, we showed here that HBx variants have retained their ability to counteract Smc5/6 and thus to activate cccDNA transcription although they tend to lose antiproliferative activity.

1.Introduction

Hepatitis B virus (HBV) is a widespread pathogen and one of the most important environmental risk factors in human cancer epidemiology. Despite the existence of an effective vaccine, the number of chronic HBV-carriers worldwide reaches 250 million individuals, who are at high risk of developing hepatocellular carcinoma (Buendia and Neuveut, 2015).

HBV-induced oncogenesis may involve a combination of direct and indirect effects of the virus during the multistep process of liver carcinogenesis. Liver inflammation and hepatocyte proliferation driven by host immune responses are recognized driving forces of liver cell transformation. Genetic and epigenetic alterations also result from viral DNA integration into cellular chromosomes and from prolonged expression of viral gene products. Notably, the transcriptional regulatory protein HBx encoded by the X gene is endowed with tumor promoter activity (Riviere et al., 2013).

HBV is a prototypical member of the hepadnavirus family of DNA viruses that preferentially target hepatocytes and share the unusual feature of replicating their genome via the retrotranscription of the viral pregenomic RNA (pgRNA) into a partially double stranded relaxed circular DNA (RC-DNA) intermediate. Upon infection, RC-DNA is delivered to the nucleus and converted into a covalently closed circular DNA (cccDNA) that serves as a template for the transcription of all viral RNAs (Seeger and Mason, 2015). The cccDNA contains four overlapping open reading frames (ORFs) encoding 7 main proteins including the regulatory protein HBx.

HBx is essential for virus replication (Zoulim et al., 1994), and it is believed to act primarily at the level of transcription (Keasler et al., 2007; Leupin et al., 2005; Tang et al., 2005). In the setting of infection, HBx expression was shown to be essential for HBV RNA expression through the establishment of an active chromatin state (Lucifora et al., 2011; Riviere et al., 2015a). HBx transcriptional activity depends on different mechanisms including the assembly of coactivator-transcription factor complexes that modulate transcription and chromatin (Belloni et al., 2009; Cougot et al., 2007, Cougot, 2012 #7290; Riviere et al., 2015b). HBx is also known to bind DDB1, a core subunit of the Cul4A-based ubiquitin E3 ligase complex, and this interaction is essential for virus replication and for the maintenance of HBx transcriptional activity. Through its interaction with DDB1, HBx acts as an adaptor for the E3 Cul4A/DDB1 complex, which induces the ubiquitination of one or more substrates. Recently, it has been shown that HBx induces the degradation of structural maintenance of the chromosome proteins 5/6 (Smc5/6) allowing HBV transcription

(Decorsiere et al., 2016). Besides its role on HBV transcription, HBx may play a role as a co-factor in oncogenesis. The HBx protein induces liver cancer in a few transgenic mouse models, and cooperates with oncogenes or chemical carcinogens to promote hepatocarcinogenesis (Benhenda et al., 2009a). Search for HBx variants expressed specifically in tumor tissues has revealed two major types of changes: point mutations and C-terminal truncations resulting from preferential integration of HBV at the direct repeats DR1 and DR2 sites (Sung et al., 2012). C-terminal truncated variants have lost transcriptional activity and antiproliferative activity of wild type full length HBx (FL-HBx), and have acquired novel properties such as promotion of cell growth and cooperation with oncogenes in cell transformation (Liu et al., 2012; Tu et al., 2001 ; Xu et al., 2007). Additionally, point mutations have been reported in FL-HBx sequences that can be encoded by either free or integrated HBV DNA, (Chen et al., 2005; Liu et al., 2014; Liu et al., 2008), with variable impact on HBx transcriptional and antiproliferative activities (Kwun and Jang, 2004; Lin et al., 2005; Liu et al., 2014).

Recent studies using more sensitive methods could detect cccDNA in tumor samples in addition to integrated viral DNA (Bai et al., 2013; Halgand et al., 2018; Marchio et al., 2018). While the transcriptional status of tumor cccDNA has not been precisely studied, HBV transcription and replication can occur at variable levels in tumor cells, and could be responsible for HBV reactivation after liver transplantation (Altinel et al., 2016; Bai et al., 2013; Faria et al., 2008). Our recent study has demonstrated that HBV replication occurs in a subset of tumors characterized by a weakly invasive phenotype and a specific transcriptomic signature. In support of this finding, we found genotypic differences between virus in tumor (T) and virus in non tumor (NT) in 11/63 cases, arguing that HBV replicates in tumor cells (Halgand et al., 2018).

The aim of the present study is to assess whether the HBx proteins encoded by full-length HBV DNA (cccDNA) in HCC contain specific mutations and harbor particular activities. We show that contrary to what has been shown for HBx expressed from integrated HBV DNA in tumors, most of HBx variants isolated from tumors replicating HBV retain their ability to support HBV cccDNA transcription and to degrade Smc5/6. Moreover, in parallel, they tend to lose antiproliferative activity, which can be viewed as detrimental for tumor growth, as demonstrated for HBx expressed from integrated sequences. Interestingly, some mutants have lost antiproliferative activity but can still support cccDNA transcription, suggesting that these two activities are distinct.

2. Methods

2.1. Amplification and cloning of HBx natural variants

The amplification and cloning of HBx from T and NT liver samples extracted from the French liver Biobanks network-INCa have been described previously (Halgand et al., 2018). Briefly, frozen tissues were homogenized using Precellys beads and total DNA was extracted using the MasterPure Complete DNA purification kit (Epicentre). Full-length HBV genome (3.2 kb) was first amplified as described by (Gunther et al., 1995) with sense P1(5'-CCG GAA AGC TTA TGC TCT TCT TTT TCA CCT CTG CCT AAT CAT C-3') and antisense P2 (5'-CCG GAG AGC TCA TGC TCT TCA AAA AGT TGC ATG GTG CTG GTG-3') primers and the whole X gene was then amplified using a nested-PCR assay with HBx-1262 sens (5'-GAT CCA TAC TGC GGA ACT CC-3') and antisense P2 (5'-CCG GAG AGC TCA TGC TCT TCA AAA AGT TGC ATG GTG CTG GTG-3') primers (Halgand et al., 2018). PCRs were performed using a hot-start procedure with the Expand High-Fidelity PCR System (Roche Diagnostics, France), according to the manufacturer's instructions. To study HBx originating from the complete (i.e. non-integrated) HBV episomal DNA we only analysed samples for which the internal primer set (primers HBx-1262 sens and antisense P2) was unable to amplify the X gene without preliminary amplification of the whole genome with primers P1 and P2. The nested PCR products were cloned into the pCR4 plasmid (TOPO TA cloning Kit) and sequenced (Halgand et al., 2018). 5 to 45 clones (mean: 20 ± 14) per sample were consequently sequenced bidirectionally by universal priming by GATC Biotech (Konstanz, Germany). The sequences were aligned using the Clustal module of MEGA software with a panel of complete HBV genotypes retrieved from public databases. The references are for: A genotypes: **AF090842.1**, **X02763.1**, **X51970.1**, **AY738142**, **AY934772**, **Z72478**, **AB116077**, **AM180624, FJ692554**, **GQ331048**; B genotypes: **AB033554.1**, **AF100309.1**, **AB033554**, **AF100309**, **D00330**, **AB073828, AY596111, AB010291**, **M54923**, **D00331**; C genotypes: **AB014381.1**, **AY123041.1**, **X04615.1**, **AB014381**, **AB112348**, **AY217376**, **M38636**, **X75665**, **AF241410**, **AB493840**, **M12906**; D genotypes: **M32138.1**, **X85254.1**, **X59795**, **EU594396**, **AB222709**, **AB109475**, **Z35716**, **X80925**, **X65257**, **DQ315779**; E genotypes**: X75657.1**, **AB032431.1**, **AB194947**, **AB194948**, **X75657**; F genotypes: **AB036910.1**, **AF223965.1**, **X69798.1**, **X69798**, **AY090461**, **AY090455**, **AF223964**, **DQ899147**, **DQ899150**, **AF223962**; G genotypes: **AB064310.1**, **AF160501.1**, **AF405706.1**, **AB056513**, **AF160501**, **AB064311**, **AB056515**; H genotypes: **AY090454.1**, **AY090457.1**, **AY090460.1**, **AB266536**, **AB059661**, **AB059660, AB205010**, **EF157291**, **AB179747**, **AB064315**. We then selected the more frequently represented sequence for each T and NT samples for further studies.

The N-terminal HA-tagged HBx T and NT variants from 11 liver samples were cloned at the BglII and Xho sites into pcDNA3.1 and into the lentiviral pTRIPΔU3 vector. Virions were produced by calcium phosphate transfection of HEK293T cells as previously described (Riviere et al., 2015a). Supernatants were collected 3 days after transfection and virus was purified by ultracentrifugation through a 20% (wt/vol) sucrose cushion. Virus production was normalized by measuring supernatant reverse transcriptase (RT) activity.

2.2. Cells and HBV production

HeLa and HEK 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum (FCS). HepG2 H1.3Δx cells are derived from HepG2 cells and contain an integrated 1.3 HBV genome that carries a stop codon mutation in both HBx open reading frames (Lucifora et al., 2011). HepaRG cells were grown in a standard medium: William's E medium supplemented with 10% FCS, 7 x 10-5 M hydrocortisone hemisuccinate, and 5 μg/ml insulin. For differentiation (dHepaRG), HepaRG cells were maintained for 2 weeks in standard medium then for at least 2 weeks in standard medium with 1.8% dimethylsulfoxide (DMSO) and EGF (5ng/ml) (PeproTech-Tebu France), as previously described (Gripon et al., 2002).

For production of HBV deficient of HBx expression (HBV X-), HepG2 H1.3Δx cells were grown in Williams E medium supplemented with 5% FCS, 7.10^{-5}M hydrocortisone hemisuccinate, 5 mg/ml insulin, and 2% DMSO. HBV particles were concentrated from the clarified supernatant through overnight precipitation in 5% PEG 8000 followed by centrifugation at 4°C (60 min at 5,292g). Titers of the enveloped DNA-containing viral particles were determined by immunoprecipitation with an anti-preS1 antibody (gift by C. Sureau, dilution 1/2000), followed by qPCR quantification of viral RC-DNA using RC primers: RC 5' (5'-CACTCTATGGAAGGCGGGTA-3') and RC 3' (5'-TGCTCCAGCTCCTACCTTGT-3'). Enveloped DNA-containing viral particles (vp) quantification was used to normalize for virus infection, and multiplicities of infection (MOI) were expressed as vp per cell.

2.3. Plasmids

The RSV-cyclic AMP-dependent kinase (PKA) expression construct was obtained from R. Maurer (Maurer, 1989). The pCRE-Luc reporter plasmid, which carries four consensus CRE sites, was from Stratagene. The N-terminally HA-tagged HBx (adw subtype) (HA-HBx) expression vector has been described previously (Cougot et al., 2007). pTRIP-Flag-HA-HBx plasmid was generated by cloning the BglII-KpnI fragment containing wild type Flag-HA-HBx cDNA in the BamHI-KpnI sites of the lentiviral vector pTRIPΔU3 (Benhenda et al., 2013). Plasmid encoding DDB1 fused to FLAG tag has been previously described.

2.4. HBV X- infection and trans-complementation assay

dHepaRG cells were infected with normalized amounts of HBV X- virus at MOI of 100 vp/cell, as described (Riviere et al., 2015a) . Briefly, cells were incubated over night with the inoculum in presence of 4% of PEG 8000. Seven days later, infected cells were transduced with normalized amounts of control lentiviral vector (TRIP Mock) or lentiviral vector encoding HA-tagged HBxwt or HA-tagged HBx variants. Four days after transduction, HBV transcription was analyzed by RT-qPCR. The level of HBV RNA in cells transduced with TRIP Mock was set to 1.

2.5. Quantitative RT-PCR (RT-qPCR)

Total RNA was isolated using TRIzol reagent (Invitrogen) and treated with TURBO DNase (Ambion). RNA (500 ng) was retrotranscribed using random primers and RevertAid H Minus M-MuLV reverse transcriptase (Fermentas). cDNA was amplified by quantitative PCR (qPCR) using SybrGreen PCR Master mix (Applied Biosystems) on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using standard qPCR protocol (Ducroux et al., 2014). For relative quantifications Rhot2 was used as a reference gene because of its low variation coefficient in human liver tumors and cell lines (Cairo et al., 2008). Values were calculated according to the ΔCt quantification method with ΔCt = Ct HBV – Ct Rhot2. Results are expressed as the average of at least three independent experiments. Standard error of the mean (SEM) are indicated. The primers HBV RNAall-F (TGAACCTTTACCCCGTTGCC) and HBV RNAall-R (GTATGGATCGGCAGAGGAGC) amplify all HBV transcripts (pregenomic RNA (pgRNA) as well as the 2.4 and 2.1 kb mRNA) except the 0.8 Kb transcript encoding HBx. The primers used to quantify Rhot2 were; Rhot2-F (CTGCGGACTATCTCTCCCCTC) and Rhot2-R (AAAAGGCTTTGCAGCTCCAC)

2.6. Luciferase assay

HeLa cells were co-transfected using Exgen reagent (Euromedex) with 0.5 ug pCRE-Luc reporter plasmid together with RSV-PKA (0.1 ug) and HBx plasmids as indicated. Total amounts of transfected DNA were kept constant by adding corresponding empty vectors. Cells were lysed 48 h later and assayed for luciferase activity. Since HBx is known to activate transcription of transfected episomal DNA such as the thymidine kinase-ß-galactosidase plasmid used for transfection efficiency normalization, we therefore confirmed the results by multiple independent assays.

2.7. Colony formation assay

$3x10^5$ HeLa cells were transfected with 1 µg of pcDNA vectors expressing HBx variants or with empty vector. Three days post-transfection, the cells were sub-cultured (1:10) and

selected with 500 µg/ml of geneticin. Drug-resistant colonies appearing 10-15 days later were fixed and stained with 0,1 % crystal violet.

2.8. Antibodies and reagents

Anti-tubulin (dilution 1/10000 for western blot WB) was purchased from SIGMA (Catalog number T5168), anti-Flag (dilution 1/1000 for WB) was obtained from Sigma Aldrich (Catalog number F3165), anti-HA (dilution 1:200 for immunofluorescence IF and 1/5000 for WB) was purchased from Covance (Catalog number MMS 101R), anti-Smc5 (dilution 1/500 for WB) was from Santa Cruz Biotechnology (Catalog number sc-393282), anti-HBx (dilution1/500 for WB and for IF) was from Abcam (Catalog number ab39716), anti-DDBI (dilution 1/1000 for WB) was from abcam (Catalog number ab109027). MLN4924 (Pevonedistat) was purchased from CliniSciences and used at 2 µM for 24 h hours.

2.9. Immunoprecipitation and Western blot analysis

For immunoprecipitation, cells were lysed at 4°C in lysis buffer (400 mM KCl, 20 mM Tris pH 7,5, 5 mM $MgCl_2$, 0,1% Triton X-100, 0,5 mM EDTA, 10% glycerol, 10 mM de β-mercaptoethanol, 0.5 mM PMSF) containing EDTA-free protease inhibitors cocktail (Roche). After lysis, the extracts were cleared by centrifugation (4°C, 13000 rpm, 20 min). Supernatant was then incubated with appropriate antibodies and beads for 2 h. Protein complexes bound to the beads were washed 3 times in lysis buffer and then eluted from the beads in loading buffer and analyzed by western blot. For Western blot analysis, samples were resolved by SDS-PAGE and electro-transferred to nitrocellulose membranes. After incubation with primary antibody, blots were probed with dye-conjugated secondary antibodies, and fluorescent immunoblot images were acquired using an Odyssey scanner (Li-CorBiosciences).

2.10. Immunofluorescence

HepaRG cells were grown on glass coverslips and transduced with normalized amounts of control lentiviral vector (TRIP Mock) or lentiviral vector encoding HA-tagged HBxwt or HA-tagged HBx variants. 48 h after transduction, cells were fixed with 4% paraformaldehyde (SIGMA) for 10 min at room temperature (RT). PFA-fixed cells were washed three times with PBS and permeabilized with 0.5% Triton X-100 in PBS, for 5 min at RT. The samples were blocked in 5% BSA and 10% SVF and incubated for 1 h at RT with the primary antibody. After 8 washes in PBS containing 0.1% Tween 20, cells were incubated with a secondary antibody coupled to Alexa 488 (1:200) for 1 h at RT. Cells were then washed 8 times in PBS containing 0.1% Tween 20 and incubated for 10 min with diaminido phenyl indol (DAPI) for nuclear staining. Coverslips were mounted with Vectashield (Vector Laboratories). Fluorescent images were acquired on a Zeiss Axio Imager Z2 microscope with

Pln-Apo 63X/1.4 objectives. Images were acquired with ZEN blue 2012 software (Carl Zeiss, Germany).

3. Results

3.1. Analysis of HBx variants encoded by HBV replicating virus present in tumor and non-tumor tissues

In a former study, we amplified and sequenced HBx encoded by episomal HBV DNA from 63 paired T and NT samples. To this aim, we amplified HBx sequence using nested PCR. We first amplified from total DNA extracted from paired T and NT the full-length HBV DNA using two external primers, followed by a nested PCR to amplify the HBx sequence (Gunther et al., 1995). To confirm that HBx was amplified from episomal DNA (RC-DNA or cccDNA) we also verified that HBx sequence could not be amplified by direct PCR using the internal HBx primers. Among the 63 paired NT and T we identified 11 patients showing genotypic differences between T and NT (Halgand et al., 2018). Among these 63 samples, we selected eight cases in which HBV cccDNA were present in both T and NT liver specimens and used cloning-sequencing to further study the activities of HBx from T and NT compartments. Five compartmentalized HBx samples (# 13, 29, 52, 67, 85 and 94) as well as three variants displaying the same genotype in T and NT were studied (Fig. 1). Cloning-sequencing confirmed that the main HBV strains in T and NT were of different genotypes showing that a predominant quasispecies population is unambiguously identified in each compartment (Fig. 1). Since HBx sequence overlaps with the coding region of the viral polymerase (pol), we verified that these mutations do not introduce a stop codon in the pol ORF. Moreover HBx sequence, beside the pol region, overlaps with HBV regulatory regions: the negative regulatory element (NRE), the basic core promoter (BCP) and the enhancer II. We thus cannot exclude that these mutations would impact viral replication. For example, we identified HBx variants with “hot spots mutations” such as the K130M mutation alone or in combination with V131I that leads to mutation of the basal core promoter and consequently modifies its activity, or H94Y that introduces changes in the enhancer II (Kramvis and Kew, 1999; Lin and Kao, 2015).

Analyses of these HBx variants revealed the presence of twelve recurrent amino acid mutations between T and NT, which were detected in at least three cases, including A12S, C26R, S33P, R78C, S101P, L116V, K130M (Table 1). Our data confirm the high frequency of the K130M mutation described in chronic hepatitis and in HCC tissues, and its association with V131I mutation, as seen in samples # 13 and # 67 (Hsia et al., 1996; Takahashi et al., 1998; Venard et al., 2000). These mutations also affect the basal core promoter sequence and could enhance viral replication and participate in HCC development (Hussain et al., 2009; Li et al., 2013; Lin et al., 2005; Liu et al., 2014). We also noted a high frequency of S101P mutation in HCC (4 of 9 tumoral variants), which was shown to increase the proliferative action of HBx (Kwun and Jang, 2004). In addition, we observed recurrent mutations at positions 87 and 88, a recurrent spot of mutations in HCC (Chen et al., 2005; Wang et al., 2012).

3.2. Activation of HBV X- cccDNA transcription by HBx variants.

The main role of HBx is to allow and maintain HBV cccDNA transcription upon infection (Lucifora et al., 2011). Since HBx variants have been isolated from replicating HBV in T and NT tissues, we first tested whether these variants have conserved this activity and are thus able to rescue transcription of a virus deficient for HBx expression (HBV X-) in the context of infection. Differentiated HepaRG cells (dHepaRG) were infected for 7 days with HBVX- and then transduced with a lentiviral vector coding for the different HA-tagged HBx variants. Analysis of the HBV RNA levels by qPCR four days post transduction showed that five out of the six compartmentalized tumor HBx studied, and six out of all the eight tumor HBx, have conserved the ability to rescue HBVX- (Fig. 2A and Table 1, "transactivation"). In the case of tumor #29, which contains two major HBx variants, one variant was able to rescue HBV X- while the other was deficient (Fig. 2A). However, some variants such as HBx T #52 and T#94 have reduced transcriptional activity. As expected, both HBx variants from T and NT in sample #76, which differ at only one amino acid, trans-complemented HBV X-. Finally, T HBx from samples #13 and #46 have lost the ability to activate HBV X- transcription. Although the T and NT HBx mutants displayed variable expression levels, no correlation could be found between HBx expression and transcriptional activity, and similar results were obtained using an higher dose of lentiviral vectors (data not shown), which suggests that the level of HBx expression was not responsible for the lack of transcriptional activation. Altogether, these data show that HBV replication in HCC correlates with the emergence of HBx variants that are still able to support HBV transcription.

3.3. Analysis of CREB-dependent transcriptional activity of HBx variants

HBx has been shown to interact with CREB/ATF factors and increase their activity through the recruitment of CBP/P300 and the inhibition of PP1/HDAC1 (Cougot et al., 2012; Cougot et al., 2007; Williams and Andrisani, 1995). We therefore monitored the activity of HBx variants towards CREB responsive element using luciferase assay. HeLa cells were transfected with pCRE-luc reporter and PKA expression vector together with either wild type HBx (HBxwt) or HBx variants. As shown in Fig. 2B, all HBx variants could stimulate CREB-dependent transcription, with higher activity for some variants such as #29T1.

3.4. Smc5/6 degradation

Next, we assessed whether the ability of HBx variants to activate HBV transcription is associated to their ability to degrade Smc5/6, as shown recently (Decorsiere et al., 2016). We transduced HEK 293T cells or HepaRG cells with lentiviral vectors coding for the different HA-tagged HBx variants. As shown in Fig.3 A and B, all variants that conserved the ability to activate the transcription of HBV X- induced the degradation of Smc5 (#13 NT, # 29NT, # 29T1, # 46NT, # 52T, # 52NT, #67NT, #67T, #76NT, #76 T, #85 NT, #85T, #94NT, #94T).

Variants that lost the ability to activate cccDNA transcription were unable to degrade Smc5 (# 13T, #29T2, #46T).

The following question was whether the inability of three HBx variants: # 13T, #29T2 and #46T, to induce Smc5/6 degradation correlates with the loss of interaction with DDB1. To this aim, HeLa cells were transfected with Flag-DDB1 alone or in combination with different HA-tagged HBx variants, followed by immunoprecipitation using anti-HA antibodies. As shown in Fig. 3C, Flag-DDB1 was specifically immunoprecipitated with all the HBx variants tested. Our data show that the interaction of HBx variants with DDB1 is not altered, but suggests rather that these T HBx variants have lost their ability to interact with Smc5/6.

We therefore expressed wt HBx or HBx variants: # 13T, #29T2 and #46T in HEK 293T cells treated or not with MLN4924, an inhibitor of E3 ubiquitin Cullin RING ligases in order to stabilize Smc6, and assessed their interaction with endogenous Smc6 and DDB1 using immunoprecipitation (Fig. 3D and E). Our results confirm that wt HBx and T variants interact with endogenous DDB1. They also show that mutants that do not induce Smc6 degradation lose or decrease their interaction with Smc6 compared to wt HBx that interacts with Smc6 even in cells with low level of Smc6 due to its degradation (Fig. 3D).

3.5. Characterization of the growth-suppressive activity of the HBx variants

Previous studies have shown that HBx variants isolated from integrated HBV sequences in tumor tissues tend to lose their antiproliferative activities, a phenotype observed in hepatic and non hepatic cells (Tu et al., 2001). Using an established method, we assessed the growth-suppressive activity of the HBx variants (Li et al., 2010). HeLa cells were transfected with the different HA-tagged HBx variants expression plasmids. G418-resistant colonies were stained 15 days after transfection. As shown in Fig. 4, wt HBx expression strongly inhibits colony formation compared to the empty vector. Interestingly, of the 8 NT/T samples studied, 5 HBx variants from tumor tissues lost their antiproliferative activity (Summarized in Table 1, “growth suppression”). Once again, the two major HBx variants in tumor #29 showed opposite activities. Interestingly, when compared to Fig. 2A, the three HBx variants (ie #13T, #29T2 and #46T) that have lost the ability to activate the transcription of the HBV X- and to induce Smc5/6 degradation, have also lost their antiproliferative activity. However, two variants (#67 and #94) have lost growth suppressive activity but are still able to activate cccDNA transcription, suggesting that the two activities can be separated, as previously suggested (Tu et al., 2001). Notably, out of the 6 HBx T variants that were able to support HBV cccDNA transcription, 4 have maintained the antiproliferative activity suggesting that the two activities are strongly associated probably because they are both required for HBV replication. Indeed, all the HBx variants from the NT tissues transactivate HBV cccDNA and show growth suppressive activity. Western blot analysis showed that all HBx variants were expressed at similar levels in HeLa cells. In all five T variants that had lost their

antiproliferative activity, we identified a mutation at position 101. Among then, 4 HBx T variants present an S to P mutation (Table 1). All these variants contain also a methionine at aa 130. Interestingly, Kwun and Jang have suggested that the regulation of p21 expression by HBx and consequently the antiproliferative activity of HBx is controlled by two opposite activities regulated respectively by aa101 and aa130 of HBx. The presence of a serine at position 101 instead of a proline is thus linked to the stronger induction of p21 and to the antiproliferative activity of HBx while the presence of methionine at aa 130 is associated with the repression of p21 (Kwun and Jang, 2004).

3.6. Sub-cellular localization of HBx variants

HBx is localized both in the nucleus and in the cytoplasm in accordance with its different activities (Benhenda et al., 2009b; Slagle and Bouchard, 2016). We examined the cellular localization of HBx T variants and of their NT counterparts in order to determine whether the mutations affect sub-cellular localization. Like for wt HBx, we observed a dual nuclear and cytoplasmic localisation for the different HBx variants, suggesting that the modifications of HBx activities cannot be explained by different subcellular localization (Fig. 5).

4. Discussion

The data presented here show that most cases of HBV-replicating HCC harboured mutations in the X gene that do not impact the ability of HBx to support HBV transcription, (6 out of 9 T variants retain transcriptional activity) but they tend to interfere with the HBx anti-proliferative activity (5 T variants out of 9 have lost antiproliferative activity). Importantly, we noted a strong correlation between the ability of HBx mutants to support HBV transcription in the setting of infection and their ability to induce Smc5/6 degradation. Mutants that have lost their ability to support HBV transcription are unable to induce the degradation of Smc5/6. These mutants are however still able to interact with DDB1 but they interact poorly or not at all with Smc6, suggesting that the mutation may rather affect Smc5/6 binding. While the minimal DDB1 interacting domain has been clearly identified and lies between position 88 and 100 in HBx, the regions binding Smc5/6 are still poorly defined (Li et al., 2010). Studies using HBx mutants have demonstrated that the transactivation function of HBx resides between amino acid 52 and 148 (Slagle and Bouchard, 2016). Interestingly, a deletion of 16 aa at HBx C-ter abolishes its transcriptional activity, suggesting that the C-ter domain might be important for Smc5/6 binding (Tu et al., 2001). The C-ter region adjoining DDB1 binding domain contains aa conserved among mammalian hepadnaviruses (Abdul et al., 2018). Interestingly, T HBx variants that have lost their ability to degrade Smc5/6 contain mutations in the C-terminal conserved region (Fig. 1). They however also contain changes in the region between aa 52 and aa 87 and we cannot exclude that this region also participates in

Smc5/6 binding. Further studies will be needed to delineate the domain of HBx involved in the recruitment of Smc5/6 and to test the impact of such mutations in the interaction.

We observed that T HBx variants have impaired antiproliferative activity. This feature has been reported previously for COOH-terminal deletion HBx variants but also for some variants found in tumors and containing amino acid changes (Lin et al., 2005; Tu et al., 2001). HBx has been shown to modulate cell cycle progression but the consequences vary depending of the cellular context. HBx induces cell cycle arrest at the G1/S transition, apoptosis and mitotic defects in proliferating cells (Bergametti et al., 1999; Martin-Lluesma et al., 2008; Sirma et al., 1999). On the contrary, in differentiated nonproliferating cells, HBx has no deleterious effect, but it seems to however stimulate quiescent hepatocytes to exit G0 and stall in G1. The same activity is observed when HBx is expressed from the HBV genome (Gearhart and Bouchard, 2010a, b). The emergence of such mutants may thus reflect adaptive mutations that are necessary to allow HBV replication in the tumor environment. A second non-exclusive hypothesis is that tumor development is due to oncogenic variants of HBx, which favour the transformation of infected hepatocytes. While controversial, HBx is believed to participate in cell transformation by a yet unknown mechanism but it is likely linked to its pleiotropic activities such as transcriptional activation, modulation of signal transduction pathway or degradation of cellular complex such as Smc5/6 that in turn impacts mitosis, DNA repair, response to genotoxic stress or apoptosis (Benhenda et al., 2009a; Livingston et al., 2017). Therefore, HBx might be involved in early stage of HCC development but then mutations/deletions arise in the X gene disrupting the antiproliferative activity in order to allow tumor cell expansion. A similar scenario is described for the viral protein Tax of human T-cell lymphotropic virus type 1 (HTLV-1). Tax, which plays a pivotal role in the development of adult T-cell leukemia/lymphoma (ALT), seems to be required in the early stage of oncogenesis but its expression is lost in leukemic cells of ALT. The loss of Tax expression in transformed cells is attributed to negative pressure exerted by the host immune system but also by the fact that expression of Tax induces cellular senescence (Giam and Semmes, 2016). On the other hand, HBx T mutants that have lost antiproliferative activity are believed to have acquired new oncogenic properties (Li et al., 2016; Tu et al., 2001; Xu et al., 2007). The mechanism underlying HBx tumor variants carcinogenesis has however not been elucidated but different mechanisms have been proposed. Studies have suggested that the first 50 aa at the N-terminal domain of HBx are required and sufficient for HBx-induced transformation (Gottlob et al., 1998). COOH-terminal deletions in HBx mutants have been shown to induce the expression of CENP-A by yet an unknown mechanism (Liu et al., 2012). Moreover the C-terminal domain can also be involved in cell proliferation and tumor development. Studies have shown that M130 mutation is associated with strong suppression of p21 expression (Kwun and Jang, 2004). The dual mutation K130M/V131I has been shown to increase HIF1α expression and activity (Liu et al., 2014). The emergence of point mutant variants could fine-tune the regulation of HBx activities,

keeping only activities compatible with cell proliferation and/or favouring cell growth. Further studies are needed to determine whether the T variants described here participate in cell transformation or in the maintenance of the transformed phenotype.

We observed that out of the six T variants that retained transcriptional activity, two (#94 and #67) have lost the antiproliferative activity suggesting that the two activities can be separated. The same was observed for mutants containing a C-terminal deletion of 14 aa (Tu et al., 2001). It has been shown that the interaction of HBx with DDB1 is required for both for transcriptional activation and antiproliferation activity (Leupin et al., 2005; Sitterlin et al., 2000). Recently Decorsière and collaborators have shown that HBx transcriptional activity is linked to its ability to induce the degradation of Smc5/6 through its interaction with DDB1 (Decorsiere et al., 2016). Since mutants #94 and #67 are still able to induce Smc5/6 degradation, one may hypothesise that HBx induces the degradation of additional substrate(s). Alternatively, mutations may affect the ability of HBx to interact with p53 or c-FLIP or any other pathway involved in HBx antiproliferative activity (Slagle and Bouchard, 2016). Further studies will be needed to uncover the function altered by these mutations.

Intriguingly, we observed that some replicating HBV genomes in tumors carry a transcriptionally inactive HBx protein (#13, #29, #46). While these observations i.e. HBV replication with non-functional HBx seem paradoxical, it is known that the requirement of HBx for HBV transcription is cell context-dependent. HBx is dispensable for HBV transcription in Huh7 cells while it is essential for the initiation and maintenance of transcription in HepG2 and HepaRG cells and in primary human hepatocytes (Leupin et al., 2005; Lucifora et al., 2011; Riviere et al., 2015a). It will be interesting to analyze whether Smc5/6 expression is down regulated in this HBV replicating HCC.

We previously showed that HBV-replicating HCCs are relatively well differentiated and non-invasive tumors (Halgand et al., 2018). We show here that these tumors contain transcriptionally active cccDNA that code for HBx variants with conserved transcriptional activity and ability to counteract Smc5/6. It will be interesting to investigate whether the degradation of Smc5/6 is involved in cellular transformation and more specifically, in the particular characteristics of HCC replicating HBV.

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and GF. All authors have read and approved the final manuscript. All authors declare no conflict of interest.

Legends

Fig. 1: Amino acid sequences of NT and T HBx variants in 8 cases. Top: schematic representation of full-length HBx protein. The minimal binding domain for DDB1 is shown. KD, Kunitz domain-like region; PSR, proline-serine-rich domain. Sequences shown for NT and T samples represent the major sequence. For each case, the consensus sequences for each genotype are shown above the NT and T sequences with first, the consensus sequence for the T followed by the consensus sequence for the NT sample. Consensus sequences are from the Hepatitis B Virus Database (HBV db) (Hayer et al., 2013). Arrowhead indicates mutations in T and black dot indicates subgenotype variations.

Fig. 2. Comparison of transactivating activity of T and NT HBx variants. **A**) Trans-complementation of HBV X- transcription by T and NT HBx variants. dHepaRG cells were infected with HBVX- at MOI of 100 vp/cell. Seven days later, infected cells were transduced with a control lentiviral vector (empty) or the different lentiviral vector encoding HA-tagged HBx variants as indicated. Four days after transduction**,** HBV transcription was analyzed by RT-qPCR. Transcript level in cells transduced with TRIP Mock (empty) was set to 1. HA-HBx expression was analyzed by Western blot with anti-HA antibodies. Mean ± SEM of 3 experiments are shown. *P* values were determined by Mann-Whitney test (*, $P < 0.05$).

B) Activation of CREB/ATF-dependent transcription by HBx variants. HeLa cells were co-transfected with pCRE-Luc reporter and RSV-PKA plasmids with different combinations of wild type HBx or T and NT HBx variants as indicated. Luciferase activities were determined 48 h after transfection. The basal activity of the cells co-transfected with pCRE-Luc and the PKA expression vector was set to 1. The results are the mean ± SEM of three independent experiments carried out in duplicate. *P* values were determined by Mann-Whitney test (*, $P < 0.05$).

Fig. 3. Analysis of Smc5 protein levels in cells expressing HBx NT and T variants. Whole cell extracts from HEK293 (A) or from HepaRG cells (B), transduced with lentiviruses coding the different HA-tagged HBx variants or HA-tagged HBx wt or empty vector (mock) were analyzed by Western blot for Smc5 expression. HBx level was assessed using either anti-HA antibody or anti-HBx antibody as indicated. Of note the mutations present in variant # 94 seem to affect the recognition by the HBx antibody. Tubulin was used as loading control. (C) Co-immunoprecipitation of Flag-DDB1 with indicated HA-HBx variants using anti-HA antibodies in HeLa cells. Proteins in the immune complexes were revealed by Western blot with anti-Flag and HA antibodies. (D) and (E) HEK293 cells were transfected with either HA-tagged wt HBx or indicated HA-tagged HBx variants or empty vector (mock) and treated (E) or not (D) for 24 h with NLM4924. Cell extracts were immunoprecipitated using anti-HA antibodies. DDB1, Smc6 and HA-tagged HBx were detected by Western blot. Arrowhead indicates IgG light chain. Asterisk indicates non-specific band.

Fig. 4. Growth-suppressive effect of T and NT HBx variants. HeLa cells were transfected with pcDNA vectors containing the indicated HBx variants or with empty vector. HBx expression was analyzed by Western Blot three days after transfection using anti-HA antibody. Geneticin-resistant colonies were fixed and stained with crystal violet, two weeks after plating. The data are representative of at least two independent transfection experiments.

Fig. 5. Sub-cellular localization of HBx T and NT variants. HepaRG cells were transduced with lentiviruses coding the different HA-tagged HBx variants or HA-tagged HBx wt or empty vector (mock). After 48 h, cells were fixed and immunostained with anti-HA and anti-HBx antibodies. Scale bar: 10 µm.

Table 1. Mutations in T and NT HBx sequences: the 12 recurrent positions and HBx functional activities are indicated. Variations that can account for genotype/subgenotype difference are shown in blue while mutations are shown in red. Consensus sequences for each genotype as well as subgenotypic variability were from HBVdb (Hayer et al., 2013).

type	id	genotype	aa position in HBx												growth suppression	transactivation cccDNA	Smc5/6 degradation
			12	26	31	33	78	87	88	101	116	130	132	151			
NT	13	A		R	S		C	Q	I	P		K	F	L	+	++	+
T		G		S	P		Y	H	P	F		M	Y	F	-	-	-
NT	29	D	A	C		S	R			S	L			L	+	++	+
T1		D	S	R		P	R			S	V			F	+	++	+
T2		A	S	R		P	C			P	V			L	-	-	-
NT	46	C						M		S			F		+	++	+
T		C						H		P			I		-	-	-
NT	52	E	A	C		S	R				L	M	F	F	+	++	+
T		E	S	R		P	C				V	K	Y	L	+	+	+
NT	67	D	A	C		S	R	R	M	S	L	K			+	++	+
T		A	S	R		P	C	Q	I	P	V	M			-	++	+
NT	76	A			S										+	++	+
T		A			A										+	++	+
NT	85	D		C	S				R		L				+	++	+
T		B		R	P				H		V				+	++	+
NT	94	D	A		S		R		F	S	L				+	++	+
T		A	S		A		C		I	P	V				-	+	+

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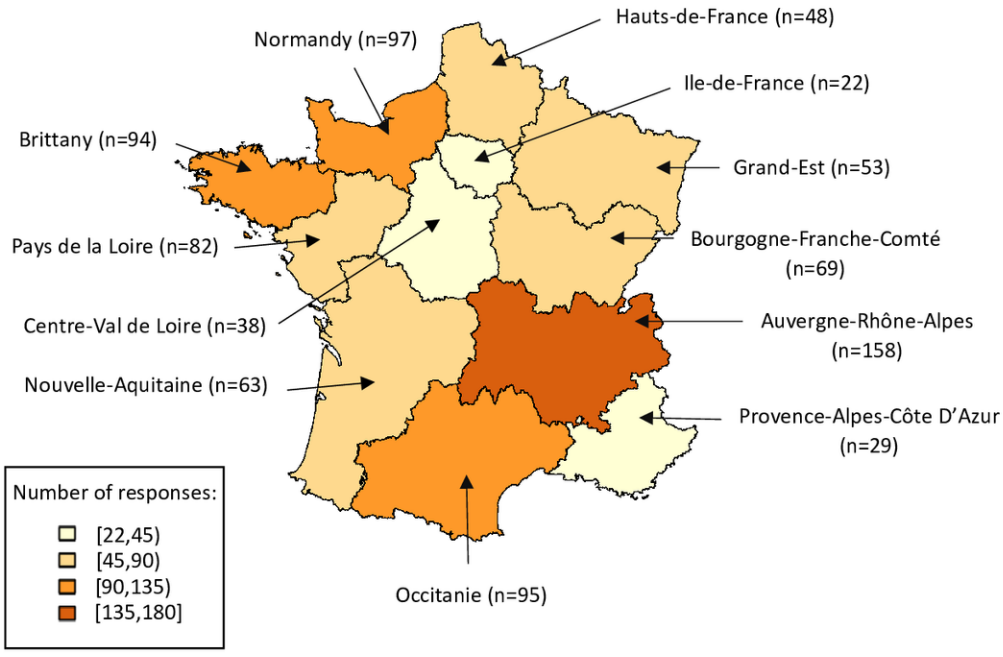
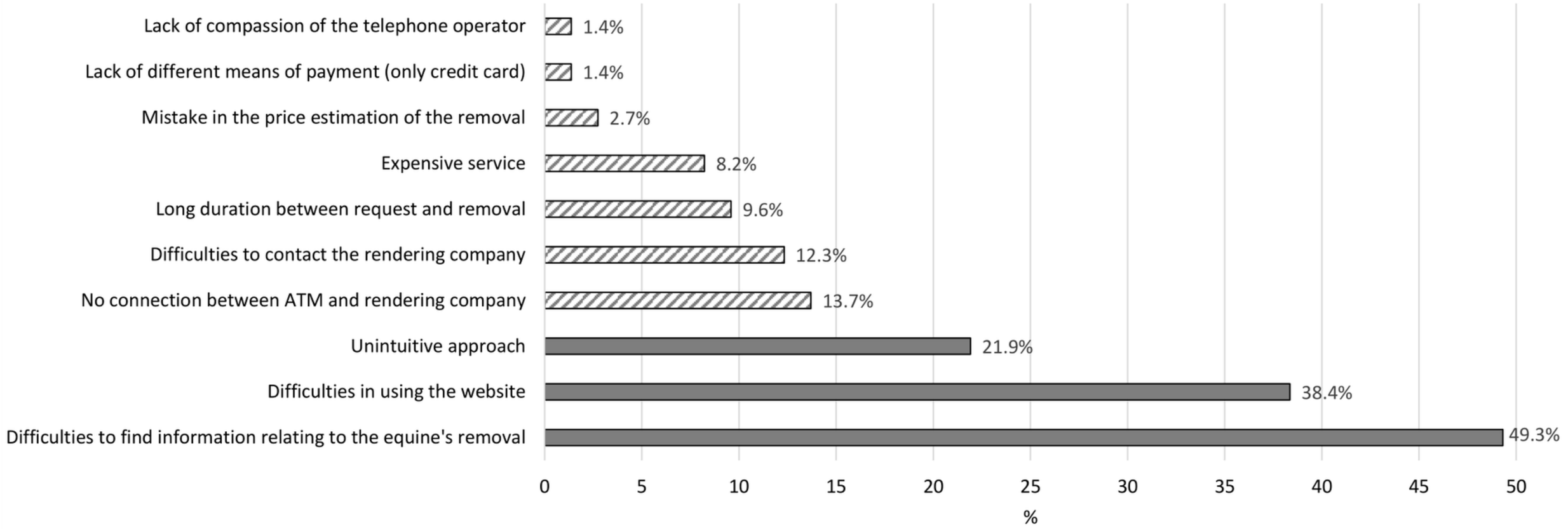
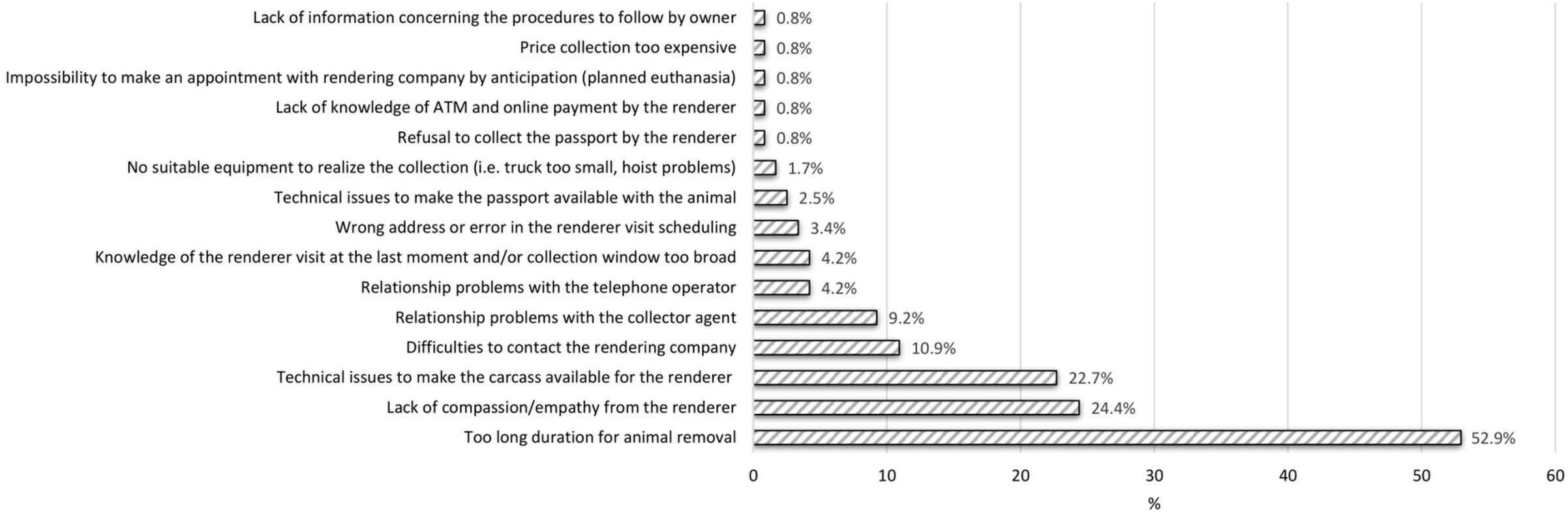
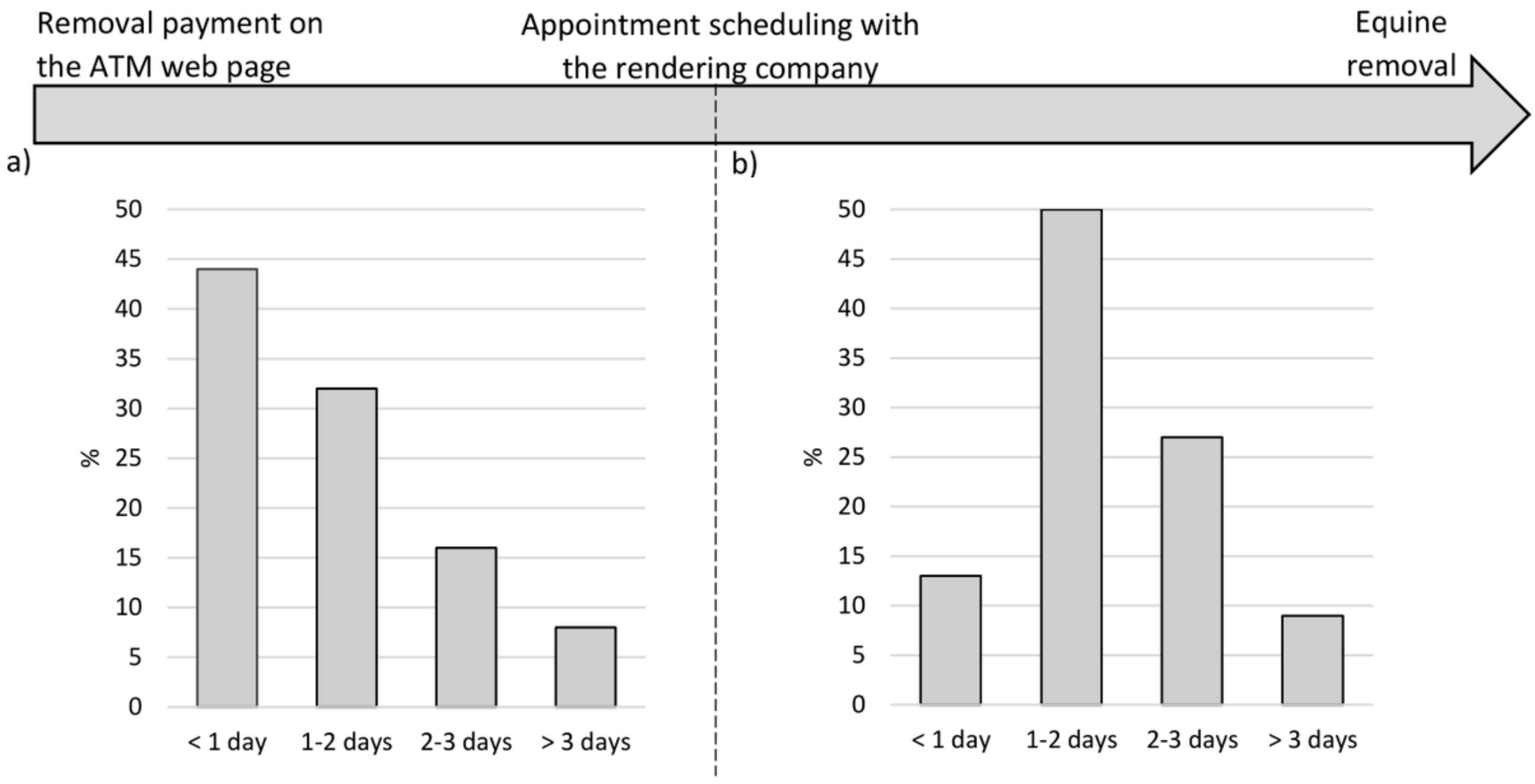
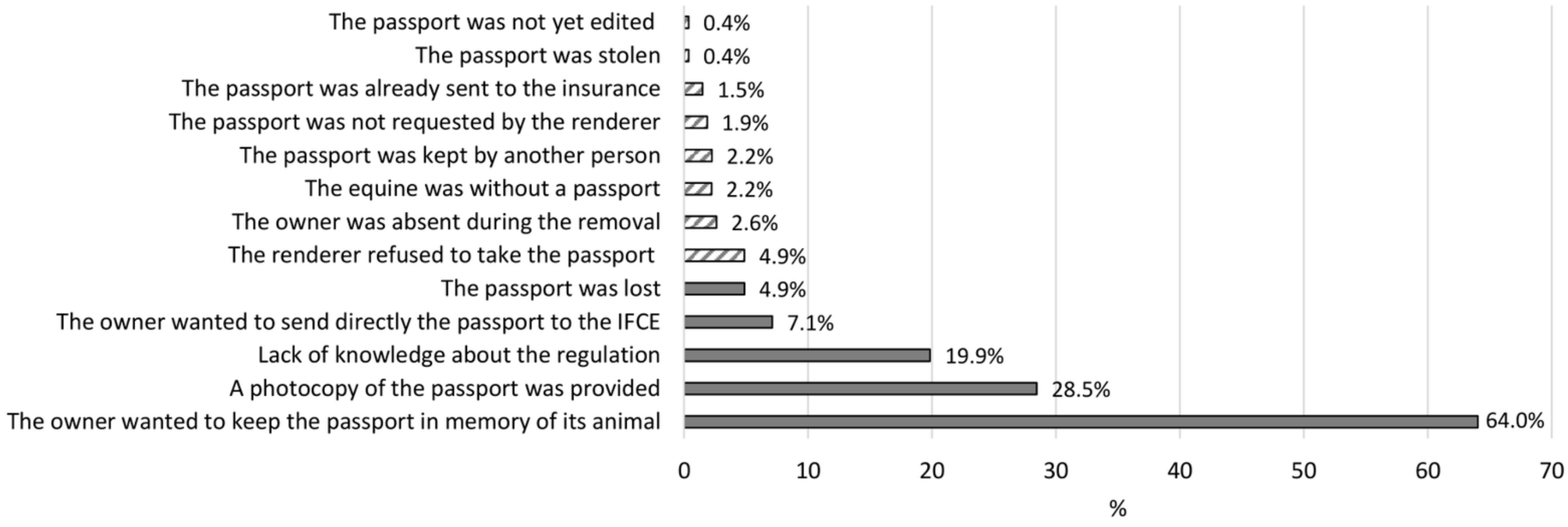

Hauts-de-France (n=48)
Normandy (n=97)
Ile-de-France (n=22)
Brittany (n=94)
Grand-Est (n=53)
Pays de la Loire (n=82)
Bourgogne-Franche-Comté (n=69)
Centre-Val de Loire (n=38)
Auvergne-Rhône-Alpes (n=158)
Nouvelle-Aquitaine (n=63)
Provence-Alpes-Côte D'Azur (n=29)
Occitanie (n=95)
Number of responses:
[22,45)
[45,90)
[90,135)
[135,180]


Lack of compassion of the telephone operator
1.4%
Lack of different means of payment (only credit card)
1.4%
Mistake in the price estimation of the removal
2.7%
Expensive service
8.2%
Long duration between request and removal
9.6%
Difficulties to contact the rendering company
12.3%
No connection between ATM and rendering company
13.7%
Unintuitive approach
21.9%
Difficulties in using the website
38.4%
Difficulties to find information relating to the equine's removal
49.3%
0
5
10
15
20
25
30
35
40
45
50
%


Lack of information concerning the procedures to follow by owner
0.8%
Price collection too expensive
0.8%
Impossibility to make an appointment with rendering company by anticipation (planned euthanasia)
0.8%
Lack of knowledge of ATM and online payment by the renderer
0.8%
Refusal to collect the passport by the renderer
0.8%
No suitable equipment to realize the collection (i.e. truck too small, hoist problems)
1.7%
Technical issues to make the passport available with the animal
2.5%
Wrong address or error in the renderer visit scheduling
3.4%
Knowledge of the renderer visit at the last moment and/or collection window too broad
4.2%
Relationship problems with the telephone operator
4.2%
Relationship problems with the collector agent
9.2%
Difficulties to contact the rendering company
10.9%
Technical issues to make the carcass available for the renderer
22.7%
Lack of compassion/empathy from the renderer
24.4%
Too long duration for animal removal
52.9%
0
10
20
30
40
50
60
%


Removal payment on the ATM web page
Appointment scheduling with the rendering company
Equine removal
a)
b)
50
45
40
35
30
25
20
15
10
5
0
%
< 1 day
1-2 days
2-3 days
> 3 days


The passport was not yet edited
0.4%
The passport was stolen
0.4%
The passport was already sent to the insurance
1.5%
The passport was not requested by the renderer
1.9%
The passport was kept by another person
2.2%
The equine was without a passport
2.2%
The owner was absent during the removal
2.6%
The renderer refused to take the passport
4.9%
The passport was lost
4.9%
The owner wanted to send directly the passport to the IFCE
7.1%
Lack of knowledge about the regulation
19.9%
A photocopy of the passport was provided
28.5%
The owner wanted to keep the passport in memory of its animal
64.0%
0
10
20
30
40
50
60
70
%